10/009919

U.S. Application No. Pending	International Application No. PCT/AU00/00439	Attorney Docket No. **DAVI151.001APC**

Date: November 13, 2001

JC10 Rec'd PCT/PTO 13 NOV 2001

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371

International Application No.:	PCT/AU00/00439
International Filing Date:	May 11, 2000
Priority Date Claimed:	May 13, 1999
Title of Invention:	LAWSONIA DERIVED GENE AND RELATED HEMOLYSIN POLYPEPTIDES, PEPTIDES AND PROTEINS AND THEIR USES
Applicant(s) for DO/EO/US:	Michael Panaccio, Everett Lee Rosey, Detlef Hasse, Robert Gerard Ankenbauer

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.

2. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).

3. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
   (X) A copy of Form PCT/IPEA/402 is enclosed.

4. (X) A copy of the International Application as filed (35 USC 371(c)(2))

   a) () is transmitted herewith (required only if not transmitted by the International Bureau).
   b) (X) has been transmitted by the International Bureau.
   c) (X) a copy of Form PCT/1B/308 is enclosed.
   d) () is not required, as the application was filed in the United States Receiving Office (RO/US).

5. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))

   a) () are transmitted herewith (required only if not transmitted by the International Bureau).
   b) () have been transmitted by the International Bureau.
   c) () have not been made; however, the time limit for making such amendments has NOT expired.
   d) (X) have not been made and will not be made.

6. (X) A copy of the International Preliminary Examination Report with any annexes thereto, such as any amendments made under PCT Article 34.

7. (X) A FIRST preliminary amendment.

8. (X) A copy of the International Application as published, including:
   (X) Publication Cover Sheet
   (X) 68 pages of disclosure and claims
   (X) 10 pages of drawings
   (X) Sequence Listing in 4 pages
   (X) International Search Report

9. (X) Disk Copy of Sequence Listing.

10. (X) PCT Request

11. (X) A return prepaid postcard.

12. (X) The following fees are submitted:

				FEES
	BASIC FEE			$1,040
CLAIMS	**NUMBER FILED**	**NUMBER EXTRA**	**RATE**	
Total Claims	43 - 20 =	23 ×	$18	$414
Independent Claims	10 - 3 =	7 ×	$84	$588
	TOTAL OF ABOVE CALCULATIONS		$2042	
	TOTAL NATIONAL FEE ENCLOSED			$2042

13. (X) The fee for later submission of the signed oath or declaration set forth in 37 CFR 1.492(e) will be paid upon submission of the declaration.

14. (X) A check in the amount of $2042 to cover the above fees is enclosed.

15. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Daniel E. Altman
Reg. No. 34,115
Customer No. 20,995

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DAVI151.001APC

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Panaccio, et al.	Group Art Unit Unknown
Int'l. Appl. No.:		PCT/AU00/00439	
Int'l Filing Date	:	May 11, 2000	
For	:	LAWSONIA DERIVED GENE AND RELATED HEMOLYSIN POLYPEPTIDES, PEPTIDES AND PROTEINS AND THEIR USES	
Examiner	:	Unknown	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Preliminary to Examination on the merits, please amend the above-captioned patent application as follows:

IN THE SPECIFICATION

On page 1, after the Title of the Invention (on line 1) and before the "Field of the Invention" (on line 4) please insert the following: --This is the U.S. National phase under 35 U.S.C. §371 of International application PCT/AU00/00439, filed May 11, 2000, and claim priority to U.S.Provisional Application 60/134022, filed May 13, 1999, both of which are herein incorporated by reference.--.

IN THE CLAIMS

Please cancel Claims 5, 9, 12, 15, and 16.

Please replace the remaining claims as follows:

1. **(Amended)** An isolated or recombinant immunogenic polypeptide comprising a *Lawsonia spp.* hemolysin polypeptide, a variant, or a truncated variant thereof, wherein said

variant or truncated variant mimics or cross-reacts with a B-cell or T-cell epitope of *Lawsonia spp.* hemolysin polypeptide.

2. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 1 wherein said polypeptide elicits the production of antibodies against *Lawsonia* spp. when administered to an avian or porcine animal.

3. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 1 which confers a protective immune response against *Lawsonia spp.* when administered to an avian or porcine animal.

4. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 1 wherein the *Lawsonia spp. is L. intracellularis.*

6. **(Amended)** An isolated or recombinant immunogenic polypeptide comprising:

(i) a peptide, oligopeptide or polypeptide comprising an amino acid sequence which has at least about 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1; or

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence having at least about 50% sequence identity to amino acid residues 1 to 50 of SEQ ID NO:1; or

(iii) a homologue or derivative of (i) or (ii) which mimics a B-cell or T-cell epitope of a *Lawsonia spp.* hemolysin polypeptide.

7. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 6 wherein said polypeptide elicits the production of antibodies against *Lawsonia* spp. in a porcine or avian animal.

8. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 6 wherein said polypeptide confers a protective immune response against *Lawsonia spp.* in a porcine or avian animal.

10. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 8, wherein said protective immune response is induced in a porcine animal.

11. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 6 wherein the *Lawsonia spp.* is *L. intracellularis.*

13. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 6 comprising the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195).

14. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 13 consisting essentially of the amino acid sequence of SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195).

17. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 6 **[that comprises]**comprising amino acid residues about 1 to about 50 of SEQ ID NO:1 **[and is capable of eliciting]**wherein said polypeptide elicits the production of antibodies against *Lawsonia intracellularis* when administered to an avian or porcine animal.

19. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 17 which induces a protective immune response against *Lawsonia intracellularis* in a porcine or avian animal.

20. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 19 which induces a protective immune response against *Lawsonia intracellularis* in a porcine animal.

21. **(Amended)** A vaccine composition for the prophylaxis or treatment of infection of an animal by *Lawsonia* spp., said vaccine composition comprising an effective amount of an immunogenic component comprising an isolated or recombinant polypeptide having at least about 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1 or at least about 50% sequence identity to amino acid residues 1 to 50 of SEQ ID NO:1 or an immunogenic homologue, or derivative thereof which is immunologically cross-reactive with *Lawsonia intracellularis;* and one or more carriers, diluents or adjuvants suitable for veterinary or pharmaceutical use.

23. **(Amended)** The vaccine composition according to claim 20 wherein the isolated or recombinant polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195).

27. **(Amended)** A combination vaccine composition for the prophylaxis or treatment of the infection of an animal by *Lawsonia* spp., said vaccine composition comprising:

(i) a first immunogenic component comprising an isolated or recombinant polypeptide having at least about 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1 or at least about 50% sequence identity to amino acid residues 1 to 50 of SEQ ID NO:1or an immunogenic homologue or derivative thereof which is immunologically cross-reactive with *Lawsonia intracellularis;*

(ii) a second immunogenic component comprising an antigenic *L. intracellularis* peptide, polypeptide or protein; and

(iii) one or more carriers, diluents or adjuvants suitable for veterinary or pharmaceutical use.

28. **(Amended)** A vaccine vector comprising a polynucleotide that encodes the immunogenic polypeptide of SEQ ID NO: 1, a homologue or a variant thereof operably linked to a promoter.

29. **(Amended)** The vaccine vector of claim 28 wherein the polynucleotide comprises SEQ ID N0: 2 a homologue, or derivative thereof which has at least about 70% sequence identity thereto.

31. **(Amended)** A polyclonal or monoclonal antibody molecule that binds specifically to a hemolysin polypeptide or a derivative of an hemolysin polypeptide from *Lawsonia s*pp. wherein said derivative has at least about 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1.

34. **(Amended)** A method of diagnosing the infection of a porcine or avian animal by *Lawsonia intracellularis* or a microorganism that is immunologically cross-reactive thereto, said method comprising the steps of: contacting a biological sample derived from said animal with the antibody molecule of claim 31 for a time and under conditions sufficient for an antigen:antibody complex to form, and detecting said complex formation.

35. **(Amended)** The method of claim 34 wherein the biological sample is selected from the group consisting of serum, lymph nodes, ileum, caecum, small intestine, large intestine, faeces or a rectal swab derived from a porcine animal.

36. **(Amended)** A method of identifying a previous or current infection with *Lawsonia intracellularis* or a microorganism that is immunologically cross-reactive thereto, said method comprising:

contacting blood or serum from said animal with the immunogenic polypeptide of claim 1 for a time and under conditions sufficient for an antigen: antibody complex to form; and detecting said complex formation.

37. **(Amended)** An isolated polynucleotide encoding a peptide, oligopeptide or polypeptide selected from the group consisting of:

(i) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which has at least about 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which has at least about 50% sequence identity to amino acid residue 1 to about amino acid residue 50 of SEQ ID NO:1; and

(iii) a homologue or derivative of (i) or (ii) which mimics a B-cell or T-cell epitope of or confers immunity against a *Lawsonia spp* when injected into an animal.

38. **(Amended)** The isolated polynucleotide of claim 37, wherein the peptide, oligopeptide or polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195), or about amino acid residue 1 to about amino acid residue 50 thereof, or a B-cell epitope or T-cell epitope thereof.

39. **(Amended)** The isolated polynucleotide of claim 38 comprising SEQ ID NO: 2, a complement or variant thereof.

40. **(Amended)** The isolated nucleic acid molecule of claim 39 consisting essentially of the nucleotide sequence of SEQ ID NO: 2 or a variant thereof.

41. **(Amended)** The isolated polynucleotide of claim 38 encoding from about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO:2 or a variant thereof.

42. **(Amended)** The isolated polynucleotide of claim 41 consisting essentially of that portion which encodes about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO:2 or a variant thereof.

43. **(Amended)** A method of detecting *Lawsonia intracellularis* or *Lawsonia spp* in a biological sample from a porcine or avian animal subject, said method comprising:

hybridizing one or more probes or primers from SEQ ID NO: 2 or a complement thereto to said sample; and detecting said hybridization .

44. **(Amended)** The method of claim 43 wherein the biological sample is selected from the group consisting of: serum, lymph nodes, ileum, caecum, small intestine, large intestine, faeces and a rectal swab from a porcine animal.

45. **(Amended)** The method of claim 44 wherein the detection is by any nucleic acid based hybridization or amplification reaction.

46. **(Amended)** A probe or primer comprising least about 15 contiguous nucleotides from SEQ ID NO: 2 or the complement thereof.

47. **(Amended)** The plasmid pALK13 (ATCC Accession No. 207196).

48. **(Amended)** The combination vaccine according to claim 27 wherein the second immunogenic component is selected from the group consisting of OmpH, FIgE, hemolysin and autolysin.

REMARKS

The claims have been amended to more clearly recite the claimed invention under United States patent practice. Claims 5, 9, 12, 15, and 16 have been deleted. As a result of the amendment, Claims 1-4, 6-8, 10-11, 13-14, 17-39 are presented for examination.

The changes made to the claims by the current amdnemnt, including **[deletions]** and additions, are shown on an attached sheet entitiled **VERSION WITH MARKINGS TO SHOW CHANGES MADE**, which follows the signature page of this Amendment.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 13 Nov. 2001

By: Daniel Altman

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Int'l. Appl. No. : PCT/AU00/00439
Date : May 11, 2000

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION

On page 1, after the Title of the Invention (on line 1) and before the "Field of the Invention" (on line 4) please insert the following: --This is the U.S. National phase under 35 U.S.C. §371 of International application PCT/AU00/00439, filed May 11, 2000, and claim priority to U.S.Provisional Application 60/134022, filed May 13, 1999, both of which are herein incorporated by reference.--.

IN THE CLAIMS

Claims 5, 9, 12, 15, and 16 have been cancelled.

1. **(Amended)** An isolated or recombinant immunogenic polypeptide **[which comprises, mimics or cross-reacts with a B-cell or T-cell epitope of]**comprising a *Lawsonia spp.* hemolysin polypeptide, a variant, or a truncated variant thereof, wherein said variant or truncated variant mimics or cross-reacts with a B-cell or T-cell epitope of *Lawsonia spp.* hemolysin polypeptide.

2. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 1 **[capable of eliciting]**wherein said polypeptide elicits the production of antibodies against *Lawsonia* spp. when administered to an avian or porcine animal.

3. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 1 **[capable of conferring]**which confers a protective immune response against *Lawsonia spp.* when administered to an avian or porcine animal.

4. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim **[2]**1 wherein the *Lawsonia spp. is L. intracellularis.*

6. **(Amended)** An isolated or recombinant immunogenic polypeptide **[selected from the following]**comprising:

(i) a peptide, oligopeptide or polypeptide **[which comprises]**comprising an amino acid sequence which has at least about 70% sequence identity **[overall]** to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence having at least about 50% sequence identity **[overall]** to amino acid residues 1 to 50 of SEQ ID NO:1; or

(iii) a homologue**[, analogue]** or derivative of (i) or (ii) which mimics a B-cell or T-cell epitope of a *Lawsonia spp.* hemolysin polypeptide.

7. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 6 wherein said polypeptide **[capable of eliciting]**elicits the production of antibodies against *Lawsonia* spp. in a porcine or avian animal.

8. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim **[7]**6 wherein said polypeptide **[capable of conferring]**confers a protective immune response against *Lawsonia spp.* in a porcine or avian animal.

10. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim **[9]**8, **[capable of inducing humoral immunity against *Lawsonia* spp.]**wherein said protective immune response is induced in a porcine animal.

11. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim **[8]**6 wherein the *Lawsonia spp.* is *L. intracellularis.*

13. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 6 **[that comprises]**comprising the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195)**[and is capable of eliciting the production of antibodies against *Lawsonia intracellularis* when administered to an avian or porcine animal]**.

14. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 13 **[that consists]**consisting essentially of the amino acid sequence of SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195).

17. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 6 **[that comprises]**comprising amino acid residues about 1 to about 50 of SEQ ID NO:1 **[and is capable of eliciting]**wherein said polypeptide elicits the production of antibodies against *Lawsonia intracellularis* when administered to an avian or porcine animal.

19. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 17 **[capable of inducing]**which induces a protective immune response against *Lawsonia intracellularis* in a porcine or avian animal.

20. **(Amended)** The isolated or recombinant immunogenic polypeptide of claim 19 **[capable of inducing]**which induces a protective immune response against *Lawsonia intracellularis* in a porcine animal.

21. **(Amended)** A vaccine composition for the prophylaxis or treatment of infection of an animal by *Lawsonia* spp., said vaccine composition comprising an effective amount of an immunogenic component **[which comprises]**comprising an isolated or recombinant polypeptide having at least about 70% sequence identity **[overall]** to the amino acid sequence set forth in SEQ ID NO: 1 or at least about 50% sequence identity **[overall]** to amino acid residues 1 to 50 of SEQ ID NO:1 or an immunogenic homologue, **[analogue]** or derivative thereof which is immunologically cross-reactive with *Lawsonia intracellularis;* and one or more carriers, diluents or adjuvants suitable for veterinary or pharmaceutical use.

22. **(Amended)** The vaccine composition according to claim 21 wherein the *Lawsonia spp*, is *L. intracellularis.*

23. **(Amended)** The vaccine composition according to claim **[22]**20 wherein the **[immunogenic component comprises an]** isolated or recombinant polypeptide **[that]** comprises the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195).

24. **(Amended)** The vaccine composition of claim 23, wherein the **[immunogenic component]**isolated or recombinant polypeptide consists essentially of the amino acid sequence of SEQ ID NO: 1.

27. **(Amended)** A combination vaccine composition for the prophylaxis or treatment of the infection of an animal by *Lawsonia* spp., said vaccine composition comprising:

(i) a first immunogenic component **[which comprises]**comprising an isolated or recombinant polypeptide having at least about 70% sequence identity **[overall]** to the amino acid sequence set forth in SEQ ID NO: 1 or at least about 50% sequence identity **[overall]** to amino acid residues 1 to 50 of SEQ ID NO:1or an immunogenic homologue**[, analogue]**or derivative thereof which is immunologically cross-reactive with *Lawsonia intracellularis;*

(ii) a second immunogenic component comprising an antigenic *L. intracellularis* peptide, polypeptide or protein; and

(iii) one or more carriers, diluents or adjuvants suitable for veterinary or pharmaceutical use.

28. **(Amended)** A vaccine vector **[that comprises, in an expressible form, an isolated nucleic acid molecule having a nucleotide sequence]**comprising a polynucleotide that encodes the **[an isolated or recombinant]** immunogenic polypeptide **[which comprises the amino acid sequence set forth in]**of SEQ ID NO: 1, a homologue or a variant thereof**[, such that said immunogenic polypeptide is expressible at a level sufficient to confer immunity against *Lawsonia* spp., when administered to a porcine or avian animal]**operably linked to a promoter.

29. **(Amended)** The vaccine vector of claim 28 wherein the **[immunogenic polypeptide is expressed using the steps of:**

(i) placing an isolated nucleic acid molecule which comprises the nucleotide sequence set forth in]polynucleotide comprises SEQ ID N0: 2 **[or degenerate variant,]** a homologue, **[analogue]** or derivative thereof which has at least about 70% sequence identity thereto**[, in operable connectin with a promoter sequence;**

(ii) introducing the isolated nucleic acid molecule and promoter sequence of step (a) into the vaccine vector; and

(iii) incubating, growing, or propagating the vaccine vector for a time and under conditions sufficient for expression of the immunogenic polypeptide encoded by said nucleic acid molecule to occur].

31. **(Amended)** A polyclonal or monoclonal antibody molecule that **[is capable of binding]**binds specifically to a hemolysin polypeptide or a derivative of an hemolysin polypeptide **[that is derived]** from *Lawsonia s*pp. **[and]**wherein said derivative has at least about 70% sequence identity **[overall]** to the amino acid sequence set forth in SEQ ID NO: 1.

34. **(Amended)** A method of diagnosing the infection of a porcine or avian animal by *Lawsonia intracellularis* or a microorganism that is immunologically cross-reactive thereto, said method comprising the steps of: contacting a biological sample derived from said animal

with the antibody molecule of claim 31 for a time and under conditions sufficient for an antigen:antibody complex to form, and **[then]** detecting said complex formation.

35. **(Amended)** The method of claim 34 wherein the biological sample **[comprises whole]**is selected from the group consisting of serum, lymph nodes, ileum, caecum, small intestine, large intestine, faeces or a rectal swab derived from a porcine animal.

36. **(Amended)** A method of identifying **[whether or not a porcine or avian animal has suffered from a past infection, or is currently infected,]**a previous or current infection with *Lawsonia intracellularis* or a microorganism that is immunologically cross-reactive thereto, said method comprising:

contacting blood or serum **[derived]** from said animal with the immunogenic polypeptide of claim 1 for a time and under conditions sufficient for an antigen: antibody complex to form; and **[then]** detecting said complex formation.

37. **(Amended)** An isolated **[nucleic acid molecule which comprises a sequence of nucleotides that encodes, or is complementary to a nucleic acid molecule that encodes,]**polynucleotide encoding a peptide, oligopeptide or polypeptide selected from the **[following]** group consisting of:

(i) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which has at least about 70% sequence identity **[overall]** to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which **[comprises an amino having]**has at least about 50% sequence identity **[overall]** to amino acid residue 1 to about amino acid residue 50 of SEQ ID NO:1; **[or]**and

(iii) a homologue**[, analogue]** or derivative of (i) or (ii) which mimics a B-cell or T-cell epitope of or confers immunity against a *Lawsonia spp* when injected into an animal.

38. **(Amended)** The isolated **[nucleic acid molecule]**polynucleotide of claim 37, wherein the peptide, oligopeptide or polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195), or about amino acid residue 1 to about amino acid residue 50 thereof, or a B-cell epitope or T-cell epitope thereof.

39. **(Amended)** The isolated **[nucleic acid molecule]**polynucleotide of claim 38 comprising **[the nucleotide sequence set forth in]** SEQ ID NO: 2, **[or]** a **[complementary nucleotide sequence thereto, or a degenerate]**complement or variant thereof.

40. **(Amended)** The isolated nucleic acid molecule of claim 39 consisting essentially of the nucleotide sequence of SEQ ID NO: 2 or a **[degenerate]** variant thereof.

41. **(Amended)** The isolated **[nucleic acid molecule]**polynucleotide of claim 38 **[comprising that portion of the nucleotide sequence of SEQ ID NO:2, or a degenerate variant thereof, which encodes]**encoding from about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO:2 or a variant thereof.

42. **(Amended)** The isolated **[nucleic acid molecule]**polynucleotide of claim 41 consisting essentially of that portion **[of the nucleotide sequence of SEQ ID NO:2, or a degenerate variant thereof,]** which encodes about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO:2 or a variant thereof.

43. **(Amended)** A method of detecting *Lawsonia intracellularis* or **[related microorganism]***Lawsonia spp* in a biological sample **[derived]** from a porcine or avian animal subject, said method comprising: **[the steps of]**

[hybridising]hybridizing one or more probes or primers **[derived from the nucleotide sequence set forth in]** from SEQ ID NO: 2 or a **[complementary nucleotide sequence]**complement thereto to said sample; and **[then]** detecting said hybridization **[hybridisation using a detection means]**.

44. **(Amended)** The method of claim 43 wherein the biological sample **[comprises whole]**is selected from the group consisting of: serum, lymph nodes, ileum, caecum, small intestine, large intestine, faeces **[or]**and a rectal swab **[derived]** from a porcine animal.

45. **(Amended)** The method of claim 44 wherein the detection **[means comprises]**is by any nucleic acid based **[hybridisation]**hybridization or amplification reaction.

46. **(Amended)** A probe or primer **[having at]**comprising least about 15 contiguous nucleotides **[in length derived]** from SEQ ID NO: 2 or **[a complementary nucleotide sequence thereto]** the complement thereof.

47. **(Amended)** **[A]**The plasmid **[designated]** pALK12 (ATCC Accession No. 207195).

48. **(Amended)** The combination vaccine according to claim 27 wherein the second immunogenic component **[comprises an antigenic *L. intracellularis* peptide, polypeptide or protein selected from the group consisting of]**<u>is selected from the group consisting of</u> SodC, OmpH, FIgE, and autolysin.

DAVI151.001APC

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Panaccio, et al.	Group Art Unit Unknown
Int'l. Appl. No.	:	10/009,919	
Filing Date	:	November 13, 2001	
For	:	LAWSONIA DERIVED GENE AND RELATED HEMOLYSIN POLYPEPTIDES, PEPTIDES AND PROTEINS AND THEIR USES	
Examiner	:	Unknown	

SUPPLEMENTAL
PRELIMINARY AMENDMENT

United States Patent and Trademark Office
PO Box 2327
Arlington, VA 22202

Dear Sir:

Preliminary to Examination on the merits, please amend the above-captioned patent application as follows:

IN THE CLAIMS

Please amend claim 24 as follows:

24. **(Amended)** The vaccine composition of claim 23, wherein the isolated or recombinant polypeptide consists essentially of the amino acid sequence of SEQ ID NO: 1.

REMARKS

The claims have been amended to correct a mistake in the Preliminary amendment. As a result of the amendment, Claims 1-4, 6-8, 10-11, 13-14, 17-48 are presented for examination.

Int'l. Appl. No. : 10/009,919
Filing Date : November 13, 2001

The changes made to the claims by the current amendment, including **[deletions]** and additions, are shown on an attached sheet entitled **VERSION WITH MARKINGS TO SHOW CHANGES MADE**, which follows the signature page of this Amendment.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: March 14, 2002 By: Jennifer Haynes

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Int'l. Appl. No. : 10/009,919
Filing Date : November 13, 2001

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS

24. **(Amended)** The vaccine composition of claim 23, wherein the **[immunogenic component]**isolated or recombinant polypeptide consists essentially of the amino acid sequence of SEQ ID NO: 1.

DAVI151.001APC PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Panaccio, et al.	) Group Art Unit Unknown
Appl. No.	:	10/009,919	)
Filed	:	November 13, 2001	)
For	:	LAWSONIA DERIVED GENE AND RELATED HEMOLYSIN POLYPEPTIDES, PEPTIDES, AND PROTEINS	)
Examiner	:	Unknown	)

RESPONSE TO MISSING REQUIREMENTS
AND
SUPPLEMENTAL PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In response to the missing requirements mailed from the Patent and Trademark Office on February 14, 2002, and preliminary to examination on the merits, please amend the above-captioned patent application as follows:

IN THE SPECIFICATION

On page 7, fourth paragraph, lines 29-32, please replace the paragraph with the following:

--**Figure 1** is a schematic representation comparing the deduced amino acid sequence of *Lawsonia intracellularis* (L. int) hemolysin (SEQ ID NO:1) to the amino acid sequences of hemolysin polypeptides derived from *Serpulina (Treponema) hyodysenteriae* (S. hyo) (SEQ ID NO:5), *Mycobacterium tuberculosis* (M.tub) (SEQ ID NO:6), *Aquifex aeolicus* (A.aeo) (SEQ ID NO:7), *Borrelia burgdorferi* (B.bur) (SEQ ID NO:8), *Helicobacter pylori* (H. pyl) (SEQ ID NO:9), *Synechocystis sp.* (Synec) (SEQ ID NO:10) and *Bacillus subtilis* YqxC (B. sub) (SEQ

ID NO:11). Gaps have been introduced to optimise the alignment. Residues that are identical in all eight sequences appear in boldface. The *L. intracellularis* hemolysin sequence (SEQ ID NO: 1) was deduced by translation of the nucleotide sequence of the *tlyA* gene set forth in SEQ ID NO:2. --.

IN THE SEQUENCE LISTING

Please cancel from the application Original Sequence Listing pages 1-4 and substitute therefore the attached Replacement Sequence Listing pages 1-6.

REMARKS

This Response to the Notice Comply amends the Sequence listing to conform to requirements under the USPTO. The Sequence Listing and Specification were amended to add the sequences appearing in Figure 1. None of the above amendments incorporate new matter.

Enclosed herewith are: (1) a paper copy of the Replacement Sequence Listing, (2) and a computer readable version of the Replacement Sequence Listing. The Response to Notice to Comply directs entry of the paper copy of the Sequence Listing into the application. In view of the foregoing, the application is believed to fully comply with the Sequence Listing Disclosure requirements.

The changes made to the specification by the current amendment, including **[deletions]** and additions, are shown on an attached sheet entitled **VERSION WITH MARKINGS TO SHOW CHANGES MADE**, which follows the signature page of this Preliminary Amendment.

VERIFICATION UNDER 37 C.F.R.§1.821(f) & (g)

All of the sequences in the attached Sequence Listing were included in the application as filed. Pursuant to 37 C.F.R.§1.821(g), no new matter is being added herewith. As required under 37 C.F.R.§1.821(f), I hereby verify that the data on the enclosed disk and the paper copies of the Sequence Listing are identical.

Conclusion

Should there be any questions concerning this application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below. Please charge any

Appl. No. : 10/009,919
Filed : November 13, 2002

additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1449.

Respectfully submitted,

KOHN & ASSOCIATES

Dated: June 14, 2002

Kenneth I. Kohn, Reg. No. 30,955
30500 Northwestern Hwy., Suite 410
Farmington Hills, Michigan 48334
(248) 539-5050

CERTIFICATE OF MAILING

Express Mail Mailing Label No.: EV 118 534 272 US
Date of Deposit: June 14, 2002

I hereby certify that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: Commissioner of Patents, BOX PCT, Washington, D.C. 20231.

Marie M DeWitt

Appl. No. : **10/009,919**
Filed : **November 13, 2001**

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION

On page 7, fourth paragraph, lines 29-32, please replace the paragraph with the following:

--**Figure 1** is a schematic representation comparing the deduced amino acid sequence of *Lawsonia intracellularis* (L. int) hemolysin (SEQ ID NO:1) to the amino acid sequences of hemolysin polypeptides derived from *Serpulina (Treponema) hyodysenteriae* (S. hyo) (SEQ ID NO:5), *Mycobacterium tuberculosis* (M.tub) (SEQ ID NO:6), *Aquifex aeolicus* (A.aeo) (SEQ ID NO:7), *Borrelia burgdorferi* (B.bur) (SEQ ID NO:8), *Helicobacter pylori* (H. pyl) (SEQ ID NO:9), *Synechocystis sp.* (Synec) (SEQ ID NO:10) and *Bacillus subtilis* YqxC (B. sub) (SEQ ID NO:11). Gaps have been introduced to optimise the alignment. Residues that are identical in all eight sequences appear in boldface. The *L. intracellularis* hemolysin sequence (SEQ ID NO: 1) was deduced by translation of the nucleotide sequence of the *tlyA* gene set forth in SEQ ID NO:2. --.

L:\DOCS\JAH\JAH-5767.DOC
041102

SEQUENCE LISTING

<110> Panaccio, Michael
Rosey, Everett Lee
Hasse, Detlef
Ankenbauer, Robert G.

<120> LAWSONIA DERIVED GENE AND RELATED
HEMOLYSIN POLYPEPTIDES, PEPTIDES AND PROTEINS AND THEIR USES

<130> DAVI151.001APC

<140> US 10/009,919
<141> 2001-11-13

<150> PCT/AU00/00439
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<160> 11

<170> FastSEQ for Windows Version 4.0

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<213> Lawsonia intracellularis

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Leu	Ala	Glu	Ser	Arg	Glu	Gln	Ala	Lys	Arg	Leu	Ile	Met	Ala	Gly	Lys
			20					25					30		
Val	Thr	Leu	Thr	Asn	Asn	Ser	Thr	Thr	Ile	Pro	Leu	Arg	Leu	Glu	Lys
		35					40					45			
Pro	Gly	His	Lys	Tyr	Pro	Leu	Glu	Ser	Ile	Cys	Ser	Leu	Ile	Gly	Val
	50					55					60				
Glu	Arg	Phe	Val	Ser	Arg	Gly	Ala	Tyr	Lys	Leu	Leu	Thr	Ala	Leu	Asp
65					70					75					80
Phe	Phe	Lys	Ile	Asp	Val	Lys	Ser	Cys	Ile	Cys	Leu	Asp	Ala	Gly	Ala
			85						90					95	
Ser	Thr	Gly	Gly	Phe	Thr	Asp	Cys	Leu	Leu	Gln	His	Gly	Ala	Ser	Lys
		100						105					110		
Val	Tyr	Ala	Ile	Asp	Val	Gly	Lys	Gly	Gln	Leu	His	Glu	Lys	Leu	Tyr
	115						120					125			
Thr	Asn	Glu	Gln	Val	Ile	Asn	Ile	Glu	Gly	Val	Asn	Leu	Arg	Thr	Ala
	130					135					140				
Ser	Lys	Asp	Leu	Ile	Pro	Glu	Glu	Val	Asp	Ile	Leu	Thr	Ile	Asp	Val
145					150					155					160
Ser	Phe	Ile	Ser	Leu	Thr	Leu	Ile	Leu	Pro	Ser	Cys	Ile	Arg	Trp	Leu
				165					170					175	

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Lys Ala Ser Gly Ile Ilé Ile Ala Leu Ile Lys Pro Gln Phe Glu Leu
            180                 185                 190
Tyr Pro Asp Lys Ile Lys Lys Gly Val Val Lys Glu Thr Ser Leu Gln
        195                 200                 205
Tyr Glu Ala Val Glu Lys Ile Ile His Phe Cys Gln Ser Glu Leu Gly
    210                 215                 220
Leu Ile Phe Ile Gly Val Val Pro Ser Val Ile Lys Gly Pro Lys Gly
225                 230                 235                 240
Asn Gln Glu Tyr Leu Ile Tyr Leu Lys Lys Arg
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tta gca gaa agt cgt gaa caa gct aaa cga ctt att atg gca ggt aag   96
Leu Ala Glu Ser Arg Glu Gln Ala Lys Arg Leu Ile Met Ala Gly Lys
            20                  25                  30

gtt aca tta act aat aat tct aca act ata cca tta cgt ttg gaa aaa  144
Val Thr Leu Thr Asn Asn Ser Thr Thr Ile Pro Leu Arg Leu Glu Lys
        35                  40                  45

cca gga cat aaa tat cca tta gaa agt atc tgc agt tta ata ggg gta  192
Pro Gly His Lys Tyr Pro Leu Glu Ser Ile Cys Ser Leu Ile Gly Val
    50                  55                  60

gaa cgt ttt gtg agt aga gga gca tat aag cta tta act gct cta gat  240
Glu Arg Phe Val Ser Arg Gly Ala Tyr Lys Leu Leu Thr Ala Leu Asp
65                  70                  75                  80

ttt ttt aaa att gat gta aaa agt tgt att tgt ctt gat gca ggc gca  288
Phe Phe Lys Ile Asp Val Lys Ser Cys Ile Cys Leu Asp Ala Gly Ala
                85                  90                  95

tct act ggt ggg ttt aca gat tgt ctt tta caa cat gga gca tct aaa  336
Ser Thr Gly Gly Phe Thr Asp Cys Leu Leu Gln His Gly Ala Ser Lys
            100                 105                 110

gta tat gcg att gat gta ggc aaa ggt caa tta cat gag aaa ctg tat  384
Val Tyr Ala Ile Asp Val Gly Lys Gly Gln Leu His Glu Lys Leu Tyr
        115                 120                 125

act aat gaa caa gtt ata aat att gag gga gtg aat tta cgt aca gca  432
```

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Thr Asn Glu Gln Val Ile Asn Ile Glu Gly Val Asn Leu Arg Thr Ala
    130                 135                 140

tct aaa gat ctt att cct gaa gaa gta gat att tta act att gat gtt   480
Ser Lys Asp Leu Ile Pro Glu Glu Val Asp Ile Leu Thr Ile Asp Val
145                 150                 155                 160

tct ttt ata tcg ctt act ttg att tta ccg tca tgt ata cgt tgg cta   528
Ser Phe Ile Ser Leu Thr Leu Ile Leu Pro Ser Cys Ile Arg Trp Leu
                165                 170                 175

aag gct tcc gga att att att gcc tta ata aag cct caa ttt gaa tta   576
Lys Ala Ser Gly Ile Ile Ile Ala Leu Ile Lys Pro Gln Phe Glu Leu
            180                 185                 190

tat cca gat aaa ata aaa aaa ggt gta gta aaa gaa act agc ttg caa   624
Tyr Pro Asp Lys Ile Lys Lys Gly Val Val Lys Glu Thr Ser Leu Gln
        195                 200                 205

tat gaa gca gta gaa aaa att att cat ttt tgt caa tca gaa ctt gga   672
Tyr Glu Ala Val Glu Lys Ile Ile His Phe Cys Gln Ser Glu Leu Gly
    210                 215                 220

ctt ata ttt att ggt gtt gtt ccg tcg gta ata aaa ggt cca aaa gga   720
Leu Ile Phe Ile Gly Val Val Pro Ser Val Ile Lys Gly Pro Lys Gly
225                 230                 235                 240

aat caa gaa tat ctt att tac ttg aaa aaa cgt taa                   756
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                245                 250
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<213> Artificial Sequence

<220>
<223> Oligonucleotide primer RA168.

<400> 3
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<220>
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<400> 4
atagaataca aattataata ag 22

<210> 5

<211> 240
<212> PRT
<213> Serpulina (Treponema) hyodysenteriae

<400> 5

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 1               5                  10                  15
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            20                  25                  30
Ala Gln Asp Ile Ile Leu Ala Gly Cys Val Phe Val Asn Gly Lys Leu
        35                  40                  45
Glu Lys Ala Phe Val Glu Phe Gly Ile Ser Val Glu Asn Lys Ile Cys
    50                  55                  60
Leu Asp Ile Gly Val Lys Val Thr Ser Lys Ala His Lys Ile Lys Asp
65                  70                  75                  80
Thr Asp Asn Ile Ala Ser Thr Gly Gly Phe Thr Asp Cys Leu Leu Lys
                85                  90                  95
His Gly Ala Lys Lys Val Tyr Ala Leu Asp Val Gly His Asn Gln Leu
            100                 105                 110
Val Tyr Lys Leu Arg Asn Asp Asn Arg Val Glu Leu Asn Asn Leu Glu
        115                 120                 125
Phe Trp Val Thr Leu Ile Lys Pro Gln Phe Val Ser Ile Glu Asp Phe
    130                 135                 140
Asn Ala Lys Asp Ile Asn Lys Glu Met Phe Asn Asp Glu Ile Glu Ala
145                 150                 155                 160
Glu Arg Gly Asp Val Ser Lys Gly Gly Ile Ile Arg Asp Asp Ile Leu
                165                 170                 175
Arg Pro Ser Val Ile Val Ser Asp Val Ser Phe Ile Ser Ile Thr Lys
            180                 185                 190
Ile Ala Pro Ile Ile Phe Lys Glu Lys Ile Leu Asn Asn Ala Ile Ser
        195                 200                 205
Lys Ile Ile Asp Cys Gly Phe Lys Glu Val Asn Arg Thr Ile Ser Pro
    210                 215                 220
Ile Lys Gly Ala Lys Gly Asn Ile Glu Tyr Leu Ala His Phe Ile Ile
225                 230                 235                 240
```

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<213> Mycobacterium tuberculosis

<400> 6

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        35                  40                  45
Arg Ile Asp Gly Lys Leu Val Gly Ala Leu Glu Ala Phe Ala Ile Ala
    50                  55                  60
Val Ala Gly Arg Arg Cys Leu Asp Ala Gly Leu Pro Ala Val Lys Pro
65                  70                  75                  80
Ala Thr Ala Val Ser Asp Thr Thr Ala Leu Ala Ser Thr Gly Gly Phe
                85                  90                  95
```

```
Thr Glu Val Leu Leu Asp Arg Gly Ala Ala His Val Val Ala Ala Asp
            100                 105                 110
Val Gly Tyr Gly Gln Leu Ala Trp Ser Leu Arg Asn Asp Pro Arg Val
        115                 120                 125
Cys Ala Ser Arg Asp Ala Asp Ile Val Pro Leu Val Lys Pro Gln Phe
    130                 135                 140
Val Val Leu Glu Arg Thr Asn Ala Arg Gly Leu Thr Pro Glu Ala Ile
145                 150                 155                 160
Gly Gly Arg Glu Val Gly Lys Gly Gln Val Gly Pro Gly Gly Val Val
                165                 170                 175
His Asp Pro Gln Leu Arg Val Asp Leu Val Val Ala Asp Leu Ser Phe
            180                 185                 190
Ile Ser Leu Ala Thr Val Leu Pro Ala Leu Val Gly Ala Arg Ser Val
        195                 200                 205
Leu Ala Val Ala Arg Arg Ala Gln Glu Leu Gly Trp His Ser Val Gly
    210                 215                 220
Val Lys Ala Ser Pro Leu Pro Gly Pro Ser Gly Asn Val Glu Tyr Phe
225                 230                 235                 240
Leu Trp Leu Arg Thr Gln Thr Asp Arg Ala Leu Ser Ala Lys Gly Leu
                245                 250                 255
Glu Asp Ala Val His Arg Ala Ile Ser Glu Gly Pro
            260                 265
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<210> 7
<211> 258
<212> PRT
<213> Aquifex aeolicus

<400> 7

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            20                  25                  30
Ala Gln Ala Val Ile Met Ala Gly Gln Val Leu Val Asn Gly Lys Leu
        35                  40                  45
Glu Trp Ala Ile Lys Arg Phe Ser Leu Asp Leu Lys Asp Lys Val Val
    50                  55                  60
Leu Asp Val Gly Lys Val Val Asp Lys Pro Gly Tyr Arg Leu Lys Gly
65                  70                  75                  80
Asn Glu Lys Val Ser Ser Thr Gly Gly Phe Thr Asp Cys Ala Leu Gln
                85                  90                  95
His Gly Ala Lys Lys Val Tyr Ala Val Asp Val Gly Arg Gly Gln Met
            100                 105                 110
Asp Tyr Lys Leu Arg Gln Asp Pro Pro Val Phe Leu Lys Glu Asp Gly
        115                 120                 125
Leu Leu Leu Val Leu Val Lys Pro Gln Phe Val Leu Tyr Glu Glu Thr
    130                 135                 140
Asp Ala Arg Glu Leu Ser Glu Glu His Val Pro Glu Lys Glu Leu Cys
145                 150                 155                 160
Pro Arg Lys Val Lys Lys Gly Val Val Arg Glu Lys Glu His Lys Val
                165                 170                 175
Asp Leu Ile Thr Cys Asp Val Ser Phe Ile Ser Ser Thr Lys Val Leu
            180                 185                 190
Pro Asn Val Phe Lys Arg Glu Ala Leu Gln Lys Val Val Asn Phe Leu
```

```
        195                 200                 205
Lys Glu Asn Gly Phe Arg Ile Leu Gly Val Ile Lys Ser Lys Pro Lys
    210                 215                 220
Gly Thr Lys Gly Asn Glu Glu Phe Phe Val Leu Ala Gly Arg Lys Gly
225                 230                 235                 240
Glu Glu Val Asn Leu Ser Glu Ala Ile Glu Lys Ala Leu Glu Glu Val
                245                 250                 255
Val Asp
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<213> Borrelia burgdorferi

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            20                  25                  30
Glu Lys Thr Arg Lys Glu Leu Met Ile Leu Ile Leu Lys Gly Asn Ile
        35                  40                  45
Tyr Val Asn Ser Lys Leu Leu Glu Ala Leu Lys Asp Phe Glu Ile Glu
    50                  55                  60
Val Lys Asn Lys Ile Cys Val Asp Val Gly His Lys Glu Lys Asn Pro
65                  70                  75                  80
Lys Ile Leu Ile Asn Lys Thr Ser Lys Ile Ser Ser Thr Gly Gly Phe
                85                  90                  95
Thr Asp Cys Leu Leu Gln Cys Gly Ala Asn Phe Val Tyr Ser Ile Asp
            100                 105                 110
Val Gly Ile Asn Gln Leu Ser Tyr Lys Leu Arg Ile Asp Pro Arg Val
        115                 120                 125
Lys Leu Ser Asp Asn Phe Ile Ile Val Leu Ile Lys Pro Gln Phe Lys
    130                 135                 140
Val Leu Glu Arg Thr Asn Ile Phe Asp Val Thr Glu Phe Lys Ile Val
145                 150                 155                 160
Pro Glu Phe Lys Ser Leu Asn Leu Asp Ile Lys Asn Phe Asn Gly Val
                165                 170                 175
Val Ser Gly Glu Tyr Leu Asn Phe Ala Val Val Asp Val Ser Phe Arg
            180                 185                 190
Ser Ser Ile Ser Ile Cys Val Asn Leu Ile Asp Lys Ile Ile Leu Gln
        195                 200                 205
Ser Val Ile Glu Lys Phe Tyr Lys Asn Lys Leu Gln Val Lys Lys Ile
    210                 215                 220
Leu Lys Leu Lys Thr Lys Gly Lys Lys Gly Asn Gln Glu Phe Met Phe
225                 230                 235                 240
Leu Val Val Lys Ser Ser Val Leu Arg Ile Ala Ser Ser Met Gln Leu
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Leu Ser Asn Ile Glu Phe
            260
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<212> PRT
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20 25 30
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35 40 45
Gly Ala Phe Leu Glu Thr His Phe Val Asp Phe Lys Gly Lys Val Val
50 55 60
Leu Asp Val Gly Met Val Val Ser Lys Pro Ser Phe Ile Val Lys Glu
65 70 75 80
Asn Asp Lys Ile Ala Ser Lys Gly Gly Phe Ser Gln Val Ala Leu Leu
85 90 95
Lys Gly Ala Lys Arg Val Leu Cys Val Asp Val Gly Lys Met Gln Leu
100 105 110
Asp Glu Ser Leu Lys Gln Asp Lys Arg Ile Leu Ser Asp Glu Phe Leu
115 120 125
Thr Leu Phe Lys Pro Gln Phe Glu Cys Tyr Glu Glu Cys Asp Ile Arg
130 135 140
Gly Phe Lys Thr Pro Glu Thr Ile Asp Leu Ala Leu Cys Asp Val Ser
145 150 155 160
Phe Ile Ser Leu Tyr Tyr Ile Leu Glu Ala Ile Leu Pro Leu Asn Ala
165 170 175
Leu Glu Asn Phe Lys Asn His Leu Lys Thr Lys Asp Phe Gln Ile Leu
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195 200 205
Phe Ile His Phe Lys Arg Ala
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<213> Synechocystis sp.

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20 25 30
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35 40 45
Lys Val Asn Gln Lys Leu Ala Lys Ala Leu Thr Glu Phe Ala Ile Asp
50 55 60
Val Thr Gly Arg Ile Cys Leu Asp Gly Gly Gln Leu Val Asp Lys Pro
65 70 75 80
Gly Thr Leu Val Thr Met Asp Val Ala Val Ile Ser Thr Gly Gly Phe
85 90 95
Thr Asp Cys Leu Leu Gln Arg Gly Ala Thr Lys Val Tyr Gly Val Asp
100 105 110
Val Gly Tyr Gly Gln Val Ala Trp Lys Leu Arg Gln Asp Asp Arg Val

```
        115                 120                 125
Leu Leu Ala Pro Pro Arg Glu Val Val Leu Leu Val Lys Pro Gln Phe
    130                 135                 140
Ile Leu Arg Glu Arg Ala Asn Phe Arg Tyr Leu Thr Pro Gln Asp Leu
145                 150                 155                 160
Tyr Gly Asp Gln Pro Trp Glu Val Gly Arg Glu Lys Ile Gly Lys Lys
                165                 170                 175
Gly Val Val Arg Asp Ala Gln Ala Gln Pro Asp Leu Gly Val Met Asp
            180                 185                 190
Leu Ser Phe Ile Ser Leu Thr Lys Val Met Ala Pro Leu Trp Ser Ala
        195                 200                 205
Glu Ala Ile Asp Gln Val Trp Arg Ser Gly Gln Ser Leu Gly Trp Gln
    210                 215                 220
Phe Asn Gly Leu Thr Phe Ser Pro Ile Thr Gly Pro Ala Gly Asn Val
225                 230                 235                 240
Glu Tyr Leu Leu Trp Leu Ser Gln Ser Ala Val Asn Ala Ser Ala Pro
                245                 250                 255
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            260                 265                 270
Arg Asn
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<213> Bacillus subtilis YqxC

<400> 11

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            20                  25                  30
Ala Glu Thr Arg Glu Lys Ala Lys Arg Ala Ile Met Ala Gly Ile Val
        35                  40                  45
Tyr Ser Asn Glu Lys Leu Glu Lys Ala Leu Lys Glu Phe Pro Val Ser
    50                  55                  60
Val Lys Asp Lys Ile Met Ile Asp Ile Gly Asn Arg Leu Asp Lys Pro
65                  70                  75                  80
Gly Glu Lys Ile Asp Arg Asp Leu Pro Leu Ser Ser Thr Gly Gly Phe
                85                  90                  95
Thr Asp Cys Ala Leu Gln Asn Gly Ala Lys Gln Ser Tyr Ala Val Asp
            100                 105                 110
Val Gly Tyr Asn Gln Leu Ala Trp Lys Leu Arg Gln Asp Glu Arg Val
        115                 120                 125
Leu Leu Val Pro Gly Ser Asp Cys Met Ala Leu Val Lys Pro Gln Phe
    130                 135                 140
Val Val Met Glu Arg Thr Asn Phe Arg Tyr Ala Thr Pro Ala Asp Phe
145                 150                 155                 160
Thr Lys Gly Met Glu Ala Gly Arg Glu Ser Val Gly Lys Lys Gly Ile
                165                 170                 175
Val Arg Asp Pro Lys Val His Pro Glu Phe Ala Thr Ile Asp Val Ser
            180                 185                 190
Phe Ile Ser Leu Arg Leu Ile Leu Pro Val Leu Arg Thr Ala Asp Val
        195                 200                 205
```

```
Leu Lys Arg Met Ile Ser Phe Ser Ala Ala Glu Gly Tyr Ile Cys Lys
    210                 215                 220
Gly Leu Ser Phe Ser Pro Ile Thr Gly Gly Asp Gly Asn Ile Glu Phe
225                 230                 235                 240
Leu Leu Gln Phe Ala Leu Ala Gly Arg Gly Thr Gly Arg Pro Gly Thr
                245                 250                 255
Ala Gly Arg Arg Asp His Ala Arg Cys Arg Arg Ser Thr
            260                 265
```

Lawsonia derived gene and related hemolysin polypeptides, peptides and proteins and their uses

FIELD OF THE INVENTION

The present invention relates generally to therapeutic compositions for the treatment and/or prophylaxis of intestinal disease conditions in animals and birds caused or exacerbated by *Lawsonia intracellularis* or similar or otherwise related microorganism. In particular, the present invention provides a novel gene derived from *Lawsonia intracellularis* which encodes an immunogenic hemolysin peptide, polypeptide or protein. The hemolysin polypeptide described herein or a peptide homologue, analogue or derivative thereof is particularly useful as an antigen in vaccine preparation for conferring humoral immunity against *Lawsonia intracellularis* and related pathogens in animal hosts. The present invention is also directed to methods for the treatment and/or prophylaxis of such intestinal disease conditions and to diagnostic agents and procedures for detecting *Lawsonia intracellularis* or similar or otherwise related microorganisms.

GENERAL

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description. All patents, patent applications, and publications cited herein are incorporated by reference in their entirety.

Reference hereinafter to *"Lawsonia intracellularis"* or its abbreviation *"L. intracellularis"* includes all microorganisms similar to or otherwise related to this microorganism, as described by Stills (1991) or Jones *et al.*(1997) or Lawson *et al.* (1993) or McOrist *et al.* (1995).

As used herein, the word "*tlyA*", or the term "*tlyA* gene", shall be taken to refer to the gene encoding the hemolysin polypeptide of the present invention. It shall also be understood that the term "TlyA polypeptide" refers to the hemolysin polypeptide of the invention.

As used herein the term "derived from" shall be taken to indicate that a specified product, in particular a macromolecule such as a peptide, polypeptide, protein, gene or nucleic acid molecule, antibody molecule, Ig fraction, or other macromolecule, or a biological sample comprising said macromolecule, may be obtained from a particular source, organism, tissue, organ or cell, albeit not necessarily directly from that source, organism, tissue, organ or cell.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps, features, compositions and compounds.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

BACKGROUND OF THE INVENTION

The meat-producing sector of the agricultural industry is dependant upon the health of its livestock and there is a need to maintain disease-free livestock for human consumption. The industry is subject to rapid economic downturn in response to disease conditions adversely affecting livestock and the quality of meat products

derived therefrom, including those diseases which may potentially be transmitted to humans. It is important, therefore, to have well defined treatments and prophylactic and diagnostic procedures available to deal with infections or potential infections in livestock animals and humans.

Meat products derived from porcine and avian species are significant commercial products in the agriculture industry. In particular, pigs form a major component of the meat industry. However, pigs are sensitive to a wide spectrum of intestinal diseases collectively referred to as porcine proliferative enteropathy (PPE). These diseases have previously been known as intestinal adenomatosis complex (Barker and van Drumel, 1985), porcine intestinal adenomatosis (PIA), necrotic enteritis (Rowland and Lawson, 1976), proliferative haemorrhagic enteropathy (Love and Love, 1977), regional ileitis (Jonsson and Martinsson, 1976), haemorrhagic bowel syndrome (O'Neil, 1970), porcine proliferative enteritis and *Campylobacter* spp – induced enteritis (Straw, 1990).

There are two main forms of PPE: a non-haemorrhagic form represented by intestinal adenomatosis which frequently causes growth retardation and mild diarrhoea; and a haemorrhagic form, which is often fatal, represented by proliferative haemorrhagic enteropathy (PHE), where the distal small intestine lumen becomes engorged with blood. PPE has been reported in a number of animal species including pigs (McOrist *et al*, 1993), hamsters (Stills, 1991), ferrets (Fox *et al*, 1989), guinea pigs (Elwell *et al*, 1981), rabbits (Schodeb and Fox, 1990) as well as avian species (Mason *et al*, 1998).

The causative organism of PPE is a *Campylobacter*-like organism referred to herein as *"Lawsonia intracellularis"* (McOrist *et al*, 1995). The organism has also been previously referred to as *Ileal symbiont intracellularis* (Stills, 1991). PPE-like diseases in pigs may also be caused by other pathogens such as various species of *Campylobacter* (Gebhart *et al*, 1983).

Lawsonia intracellularis is an intracellular, possibly obligate intracellular, bacterium. It can only be cultured *in vitro* with tissue culture cells (Jones *et al.*, 1997; Lawson *et*

al., 1993; McOrist *et al*, 1995; International Patent Application No. PCT/US96/09576). *L. intracellularis* is located in the cytoplasm of the villus cells and intestinal crypt cells of infected animals. Pigs suffering from PPE are characterised by irregularities in the villus cells and intestinal crypt structure with epithelial cell dysplasia, wherein crypt abscesses form as the villi and intestinal crypts become branched and, fill with inflammatory cells.

PPE is a significant cost component associated with the pig industry, especially in terms of stock losses, medication costs, reduced growth rates of pigs and increased feed costs. PPE also contributes to downstream indirect costs in, for example, additional labour costs and environmental costs in dealing with antibiotic residue contamination, and in control measures to prevent the organism from being passed on or carried to other animals or humans.

Current control strategies for PPE rely on the use of antibacterials. However, such a strategy is considered to only be short to medium term, especially since governmental regulatory pressures tend to discourage animal husbandry practices which involve the use of prophylactic antibiotics. There is a need, therefore, to develop effective, safe and low cost alternatives to the use of antibiotics and, in particular, to develop vaccine preparations capable of conferring protective immunity against *Lawsonia intracellularis* infection in livestock animals.

The most effective vaccine preparations are generally comprised of a highly antigenic component, such as a peptide, polypeptide, protein or other macromolecule which is derived from the pathogenic organism against which the vaccine is directed, wherein said antigenic component produces little or no contraindications when administered to a susceptible host animal, and produces little or no antigenic cross-reactivity with desirable organisms, such as non-pathogenic organisms that are a part of the normal flora of the intestinal tract or other tissues of said host animal. In summary, an effective vaccine preparation must be immunogenic, specific and safe.

Accordingly, there is a need to identify highly immunogenic antigens produced by the

bacterium *Lawsonia intracellularis*.

International Patent Application No. PCT/AU96/00767 describes several *L. intracellularis* partial genetic sequences, and partial polypeptides encoded thereby. However, there is a need to further identify polypeptide immunogens produced by the bacterium *L. intracellularis* and immunogenic peptides derived therefrom, including those immunogens which are genus- or species-specific, for use in improved vaccine compositions. The presently-described invention provides such immunogens.

SUMMARY OF THE INVENTION

One aspect of the present invention is directed to an isolated or recombinant immunogenic polypeptide which comprises, mimics or cross-reacts with a B-cell or T-cell epitope of *Lawsonia spp*. Preferably, the isolated or recombinant immunogenic polypeptide is selected from the group consisting of the following:

(i) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which has at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence having at least about 50% sequence identity to about amino acid residues 1 to 50 of SEQ ID NO: 1 ; or

(iii) a homologue, analogue or derivative of (i) or (ii), which mimics a B-cell or T-cell epitope of *Lawsonia spp*.

In a preferred embodiment, the polypeptide comprises or consists essentially of the amino sequence of SEQ ID NO: 1, or about amino acids 1 to 50 thereof.

A further aspect of the present invention provides a vaccine composition for the prophylaxis or treatment of infection in an animal, such as a pig or bird, by *L. intracellularis* or a similar or otherwise related microorganism, said vaccine composition comprising an immunologically effective amount of an immunogenic component which comprises an isolated or recombinant polypeptide having at least about 70% overall sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, or at least

about 50% overall sequence identity to amino acid residues 1 to 50 of SEQ ID NO: 1 or an immunogenic homologue, analogue or derivative thereof which is immunologically cross-reactive with *Lawsonia intracellularis;* and one or more carriers, diluents and/or adjuvants suitable for veterinary or pharmaceutical use.

In a preferred embodiment, the polypeptide of the vaccine composition comprises or consists essentially of the amino acid sequence of SEQ ID NO: 1 or about amino acids 1 to 50 thereof.

A further aspect of the invention extends to an immunologically interactive molecule, such as an antibody or antibody fragment, which is capable of binding to the immunogenic polypeptide of the invention.

A further aspect of the invention provides a method of diagnosing infection of an animal by *Lawsonia intracellularis* or a related microorganism, said method comprising the steps of contacting a biological sample derived from said animal with an immunologically interactive molecule of the present invention for a time and under conditions sufficient for a complex, such as an antigen:antibody complex, to form, and then detecting said complex formation.

A further aspect of the present invention contemplates a method of determining whether or not an animal has suffered from a past infection, or is currently infected, by *Lawsonia intracellularis* or a related microorganism, said method comprising contacting a tissue or fluid sample, such as blood or serum derived from said animal, with the immunogenic polypeptide of the invention for a time and under conditions sufficient for a complex, such as an antigen:antibody complex, to form, and then detecting said complex formation.

A further aspect of the present invention provides an isolated nucleic acid molecule which comprises a sequence of nucleotides that encodes, or is complementary to a nucleic acid molecule that encodes, a peptide, oligopeptide or polypeptide selected from the following:

(i) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which has at least about 70% overall sequence identity to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence having at least about 50% overall sequence identity to amino acid residues 1 to 50 of SEQ ID NO: 1; or

(iii) a homologue, analogue or derivative of (i) or (ii), which mimics a B-cell or T-cell epitope of *Lawsonia spp.*

In a preferred embodiment, the isolated nucleic acid molecule comprises the nucleotide sequence set forth in SEQ ID NO: 2, or at least that portion of SEQ ID NO: 2 encoding amino acid residues 1 to 50 of SEQ ID NO: 1, or a degenerate variant thereof, or has at least about 70% sequence identity to all or a part thereof.

A still further aspect of the invention provides a diagnostic method of detecting *Lawsonia intracellularis* or related microorganism in a biological sample derived from an animal subject, said method comprising the steps of hybridising one or more polynucleotide or oligonucleotide probes or primers derived from the nucleotide sequence set forth in SEQ ID NO: 2 or a complementary nucleotide sequence thereof, or a homologue, analogue or derivative thereof, to said sample, and then detecting said hybridisation using a detection means. The detection means according to this aspect of the invention is any nucleic acid-based hybridisation or amplification reaction.

A further aspect of the invention provides an isolated probe or primer derived from SEQ ID NO: 2 or a complementary nucleotide sequence thereto.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation comparing the deduced amino acid sequence of *Lawsonia intracellularis* (L. int) hemolysin to the amino acid sequences of hemolysin polypeptides derived from *Serpulina (Treponema) hyodysenteriae* (S. hyo), *Mycobacterium tuberculosis* (M.tub), *Aquifex aeolicus* (A.aeo), *Borrelia burgdorferi*

(B.bur), *Helicobacter pylori* (H. pyl), *Synechocystis sp.* (Synec) and *Bacillus subtilis* YqxC (B. sub). Gaps have been introduced to optimise the alignment. Residues that are identical in all eight sequences appear in boldface. The *L. intracellularis* hemolysin sequence (SEQ ID NO: 1) was deduced by translation of the nucleotide sequence of the *tlyA* gene set forth in SEQ ID NO:2.

DETAILED DESCRIPTION OF THE INVENTION

In work leading up to the present invention, the inventors sought to identify immunogenic proteins of *Lawsonia intracellularis* for use in vaccines for the prophylaxis and treatment of PPE in animals, including pigs and birds.

Accordingly, one aspect of the present invention is directed to an isolated or recombinant immunogenic polypeptide which comprises, mimics or cross-reacts with a B-cell or T-cell epitope of *Lawsonia spp.*

Epitopes of *Lawsonia spp.* may be B cell epitopes or T-cell epitopes. It is well-known that antibody-binding sites (B-cell epitopes) involve linear as well as conformational epitopes (van Regenmortel, 1992). B-cell epitopes are predominantly conformational. In contrast, T-cells recognize predominantly linear epitope sequences in combination with MHC class II molecules.

A precise identification and careful selection of epitopes of *Lawsonia spp.* facilitates the development of diagnostic reagents and vaccine compositions for the effective treatment or prophylaxis of *Lawsonia* infections. Epitope identification and characterization (i.e., determination of the molecular weight, amino acid sequence, and structure of epitopes of *Lawsonia spp.*) may be performed using art-recognised techniques. For the detection of conformational epitopes, degrading and denaturing of the epitope molecule must be avoided in order to conserve the three-dimensional structure, because the antigen-antibody reaction will be diminished if the secondary structure of the epitope is altered significantly. In practice, the characterisation and isolation of linear non-conformational epitopes is easier, because any immunoreactive regions are contained within a single peptide fragment or single amino acid sequence

which is capable of being purified under a range of conditions.

Both non-conformational and conformational epitopes may be identified by virtue of their ability to bind detectable amounts of antibodies (such as IgM or IgG) from sera of animals immunised against or infected with *Lawsonia spp.* and, in particular *L. intracellularis,* or an isolated polypeptide derived therefrom or, alternatively, by virtue of their ability to bind detectable amounts of antibodies in a purified Ig fraction derived from such sera. The antibodies may be derived from or contained within pools of polyclonal sera, or may be monoclonal antibodies. Antibody fragments or recombinant antibodies, such as those expressed on the surface of a bacteriophage or virus particle, such as in a phage display library, may also be employed.

The determination of T-cell epitopes is performed by analysing the ability of the epitope peptides to induce the proliferation of peripheral blood lymphocytes or T-cell clones. The identification of T-cell epitopes is accomplished using a variety of methods as know in the art, including the use of whole and fragmented native or recombinant antigenic protein, as well as the more commonly employed "overlapping peptide" method. In the latter method, overlapping peptides which span the entire sequence of a polypeptide derived from *Lawsonia spp.* are synthesized and tested for their capacity to stimulate T-cell cytotoxic or proliferative responses *in vitro*.

Structure determination of both conformational non-linear and non-conformational linear epitopes may be performed by nuclear magnetic resonance spectroscopy (NMR) and X-ray crystallographic analysis. The determination of epitopes using X-ray techniques requires the protein-antibody complex to be crystallized, whereas NMR allows analysis of the complex in a liquid state. NMR measures the amount of amino acids as well as the neighbourhood of protons of different amino acid residues, wherein the alternating effect of two protons along the carbon backbone is characteristic of a particular epitope.

A successful method to recognize non-conformational linear epitopes is the immunoblot and in particular, the Western blot. Peptides may be generated from a

complete *Lawsonia spp.* polypeptide by digestion with site-specific proteases, such as trypsin or chymotrypsin, and the peptides generated thereby can be separated using standard electrophoretic or chromatographic procedures. For example, after electrophoresis according to molecular weight using SDS/PAGE (SDS/polyacrylamide gel electrophoresis) and/or according to isoelectric point using IEF (isoelectric focussing) or alternatively, by two-dimensional electrophoresis, the peptides can be transferred to immobilizing nylon or nitrocellulose membranes and incubated with sera raised against the intact polypeptides. Peptides that comprise immunogenic regions (i.e., B-cell or T-cell epitopes) are bound by the antibodies in the sera and the bound antibodies may be detected using secondary antibodies, such as anti-IgG antibodies, that have been labelled radioactively or enzymatically. The epitopes may then be characterised by purification based upon their size, charge or ability to bind specifically to antibodies against the intact polypeptide, using one or more techniques, such as size-exclusion chromatography, ion-exchange chromatography, affinity chromatography or ELISA amongst others. After purification of the epitope, only one band or spot should be detectable with gel electrophoresis. The N-terminal or total sequencing of the peptide offers the possibility to compare the peptide with known proteins in databases.

Several computer-driven algorithms have now been devised to search for T-cell epitopes in proteins (Margalit *et al*, 1987; Vajda and C. DeLisi, 1990; Altuvia *et al.*, 1995; Parker *et al.*1994; De Groot *et al.*,1995; Gabriel *et al*, 1995; Meister *et al.*, 1995). These algorithms search the amino acid sequence of a given protein for characteristics believed to be common to immunogenic peptides, locating regions that are likely to induce a cellular immune response *in vitro*. Computer-driven algorithms can identify regions of a *Lawsonia spp.* polypeptide that contain epitopes and are less variable among different isolates. Alternatively, computer-driven algorithms can rapidly identify regions of each isolate's more variable proteins that should be included in a multivalent vaccine.

The AMPHI algorithm (Margalit *et al.*,1987), which is based on the periodicity of T cell epitopes, has been widely used for the prediction of T-cell antigenic sites from

sequence information alone. Essentially, AMPHI describes a common structural pattern of MHC binding motifs, since MHC binding motifs (i.e., patterns of amino acids that appear to be common to most of the peptides that bind to a specific MHC molecule) appear to exhibit the same periodicity as an alpha helix. Identification of T-cell epitopes by locating MHC binding motifs in an amino acid sequence provides an effective means of identifying immunogenic epitopes in diagnostic assays.

The EpiMer algorithm (Meister *et al.*, 1995; Gabriel *et al.*, 1995; De Groot *et al.*, 1995) locates clustered MHC binding motifs in amino acid sequences of proteins, based upon the correlation between MHC binding motif-dense regions and peptides that may have the capacity to bind to a variety of MHC molecules (promiscuous or multi-determinant binders) and to stimulate an immune response in these various MHC contexts as well (promiscuous or multi-determinant epitopes). The EpiMer algorithm uses a library of MHC binding motifs for multiple class I and class II HLA alleles to predict antigenic sites within a protein that have the potential to induce an immune response in subjects with a variety of genetic backgrounds. EpiMer locates matches to each MHC-binding motif within the primary sequence of a given protein antigen. The relative density of these motif matches is determined along the length of the antigen, resulting in the generation of a motif-density histogram. Finally, the algorithm identifies protein regions in this histogram with a motif match density above an algorithm-defined cutoff density value, and produces a list of subsequences representing these clustered, or motif-rich regions. The regions selected by EpiMer may be more likely to act as multi-determinant binding peptides than randomly chosen peptides from the same antigen, due to their concentration of MHC-binding motif matches. The selection of regions that are MHC binding motif-dense increases the likelihood that the predicted peptide contains a "valid" motif, and furthermore, that the reiteration of identical motifs may contribute to peptide binding.

Additional MHC binding motif-based algorithms have been described by Parker *et al.*(1994) and Altuvia *et al.*(1995). In these algorithms, binding to a given MHC molecule is predicted by a linear function of the residues at each position, based on empirically defined parameters, and in the case of the Altuvia *et al.*(1995) algorithm,

known crystallographic structures may also be taken into consideration.

Recombinant methods offer the opportunity to obtain well characterized epitopes of high purity for the production of diagnostic reagents and epitope-specific vaccine formulations (Mohapatra *et al.*, 1995). Based upon the amino acid sequence of a linear epitope and identification of the corresponding nucleotide sequence encoding same, polymerase chain reaction (PCR) may be performed to amplify the epitope-encoding region from cDNA. After cloning and expression in a suitable vector/host system, a large amount of epitopes of high purity can be extracted. Accordingly, the present invention clearly extends to both isolated non-recombinant polypeptides and recombinant polypeptides in an impure or isolated form.

The term "polypeptide" as used herein shall be taken to refer to any polymer consisting of amino acids linked by covalent bonds and includes within its scope full-length proteins and parts or fragments thereof such as, for example, oligopeptides and short peptide sequences consisting of at least about 5 amino acid residues, preferably at least about 10 amino acid residues, more preferably at least about 12 amino acid residues, and even more preferably at least about 15 amino acid residues. Also included within the scope of the definition of a "polypeptide" are amino acid sequence variants, containing one or more preferably conservative amino acid substitutions, deletions, or insertions, which do not alter at least one essential property of said polypeptide such as, for example, its immunogenicity, use as a diagnostic reagent, or effectiveness as a peptide vaccine against *Lawsonia spp*, amongst others. Accordingly, a polypeptide may be isolated from a source in nature, or chemically synthesized. Furthermore, a polypeptide may be derived from a full-length protein by chemical or enzymatic cleavage, using reagents such as CNBr, trypsin, or chymotrypsin, amongst others.

Conservative amino acid substitutions are well-known in the art. For example, one or more amino acid residues of a native hemolysin polypeptide of the invention can be substituted conservatively with an amino acid residue of similar charge, size or polarity, with the resulting polypeptide retaining an ability to function in a vaccine or as a

diagnostic reagent as described herein. Rules for making such substitutions include those described by Dayhof (1978). More specifically, conservative amino acid substitutions are those that generally take place within a family of amino acids that are related in their side chains. Genetically-encoded amino acids are generally divided into four groups: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, and histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan; and (4) uncharged polar= glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine. Phenylalanine, tyrosine and tryptophan are also jointly classified as aromatic amino acids. One or more replacements within any particular group such as, for example, the substitution of leucine for isoleucine or valine or alternatively, the substitution of aspartate for glutamate or threonine for serine, or of any other amino acid residue with a structurally-related amino acid residue, will generally have an insignificant effect on the function of the resulting polypeptide.

The present invention is not limited by the source of the subject immunogen and clearly extends to isolated and recombinant polypeptides which are derived from a natural or a non-natural occurring source.

The term "recombinant polypeptide" as used herein shall be taken to refer to a polypeptide which is produced *in vitro* or in a host cell by the expression of a genetic sequence encoding said polypeptide, which genetic sequence is under the control of a suitable promoter, wherein a genetic manipulation has been performed in order to achieve said expression. Accordingly, the term "recombinant polypeptide" clearly encompasses polypeptides produced by the expression of genetic sequences contained in viral vectors, plasmids or cosmids that have been introduced into prokaryotic or eukaryotic cells, tissues or organs. Genetic manipulations which may be used in this context will be known to those skilled in the art and include, but are not limited to, nucleic acid isolation, restriction endonuclease digestion, exonuclease digestion, end-filling using the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase enzymes, blunt-ending of DNA molecules using T4 DNA polymerase or ExoIII enzymes, site-directed mutagenesis, ligation, and amplification reactions. As will be known to those skilled in the art, additional techniques such as nucleic acid

hybridisations and nucleotide sequence analysis may also be utilised in the preparation of recombinant polypeptides, in confirming the identity of a nucleic acid molecule encoding a desired recombinant polypeptide and a genetic construct comprising the nucleic acid molecule.

Wherein the polypeptide of the present invention is a recombinant polypeptide, it may be produced in and, if desirable, isolated from a recombinant viral vector or host cell expression system. As will be known to those skilled in the relevant art, a cell for production of a recombinant polypeptide is selected on the basis of several parameters including the genetic constructs used to express the polypeptide under consideration, as well as the stability and activity of said polypeptide. It will also be known to those skilled in the art that the stability or activity of a recombinant polypeptide may be determined, at least in part, by post-translational modifications to the polypeptide such as, for example, glycosylation, acylation or alkylation reactions, amongst others, which may vary between cell lines used to produce the recombinant polypeptide.

Accordingly, in a more particularly preferred embodiment, the present invention extends to a recombinant polypeptide or a derivative, homologue or analogue thereof as present in a virus particle, or as produced in prokaryotic or eukaryotic host cell, or in a virus or cell culture thereof.

The present invention also extends to a recombinant polypeptide according to any of the foregoing embodiments which is produced in a bacterial cell belonging to the genus *Lawsonia*, in particular a cell of *L. intracellularis*, or a culture thereof.

The term "isolated polypeptide" refers to a polypeptide of the present invention which has been purified to some extent, preferably to at least about 20% by weight of protein, preferably to at least about 50% by weight of protein, more preferably to at least about 60% by weight of protein, still more preferably to at least about 70% by weight of protein and even more preferably to at least about 80% by weight of protein or greater, from its natural source or, in the case of non-naturally-occurring polypeptides, from the culture medium or cellular environment in which it was produced. Such isolation may

be performed to improve the immunogenicity of the polypeptide of the present invention, or to improve the specificity of the immune response against that polypeptide, or to remove toxic or undesirable contaminants therefrom. The necessary or required degree of purity of an isolated polypeptide will vary depending upon the purpose for which the polypeptide is intended, and for many applications it will be sufficient for the polypeptide preparation to contain no contaminants which would reduce the immunogenicity of the polypeptide when administered to a host animal, in particular a porcine or avian animal being immunized against PPE or, alternatively, which would inhibit immuno-specific binding in an immunoassay for the diagnosis of PPE or a causative agent thereof.

The purity of an isolated polypeptide of the present invention may be determined by any means known to those skilled in the art, including the degree of homogeneity of a protein preparation as assessed by SDS/polyacrylamide gel electrophoresis, 2-dimensional electrophoresis, or amino acid composition analysis or sequence analysis.

Preferably, the polypeptide of the present invention will be substantially homogeneous or substantially free of nonspecific proteins, as assessed by SDS/polyacrylamide gel electrophoresis, 2-dimensional electrophoresis, or amino acid composition analysis or sequence analysis.

The polypeptide of the present invention can be purified for use as a component of a vaccine composition by any one or a combination of methods known to those of ordinary skill in the art, including, for example, reverse phase chromatography, HPLC, ion-exchange chromatography, and affinity chromatography, among others.

In a preferred embodiment, the isolated or recombinant polypeptide of the invention possesses hemolysin activity or is derived from a polypeptide which possesses hemolysin activity, such as, for example, *L. intracellularis* hemolysin or, alternatively, is immunologically cross-reactive with the *L. intracellularis* hemolysin polypeptide of the present invention, as determined by standard immunoassay such as RIA or ELISA, amongst others.

As used herein, the term "hemolysin activity" shall be taken to mean hemolysin enzyme activity as determined by any means known to those skilled in the art such as, for example, by the lysis of red blood cells as described by Basaraba *et al.* (1998).

In a further preferred embodiment, the isolated or recombinant polypeptide of the invention is derived from *Lawsonia spp.* and more preferably, the subject polypeptide is derived from *Lawsonia intracellularis.*

A B cell or T cell epitope of a polypeptide or a derivative, homologue or analogue thereof may comprise any combination of the following:

(i) the primary amino acid sequence of said region, known in the art as a continuous non-conformational epitope;

(ii) the secondary structure which a hemolysin polypeptide adopts, known in the art as a continuous conformational epitope;

(iii) the tertiary structure which a hemolysin polypeptide adopts in contact with another region of the same polypeptide molecule, known in the art as a discontinuous conformational epitope; or

(iv) the quaternary structure which a hemolysin polypeptide adopts in contact with a region of another polypeptide molecule, known in the art as a discontinuous conformational epitope.

Accordingly, immunogenic polypeptides or derivatives, homologues or analogues thereof comprising the same, or substantially the same primary amino acid sequence are hereinafter defined as "immunogens which comprise a B cell or T cell epitope", or similar term.

Immunogenic polypeptides or derivatives, homologues, or analogues thereof comprising different primary amino acid sequences may comprise immunologically identical immunogens, because they possess conformational B cell or T cell epitopes that are recognised by the immune system of a host species to be identical. Such immunogenic polypeptides or derivatives, homologues or analogues thereof are

hereinafter defined as "immunogens which mimic or cross-react with a B cell or T cell epitope", or similar term.

Accordingly, the present invention extends to an immunogen which comprises, mimics, or cross-reacts with a B-cell or T-cell epitope of an isolated or recombinant polypeptide according to any one of the foregoing embodiments or a derivative, homologue or analogue thereof. In a particularly preferred embodiment, the present invention provides an immunogen which comprises, mimics, or cross-reacts with a B-cell or T-cell epitope of an isolated or recombinant polypeptide which in its native form is obtainable from a species of *Lawsonia* such as, but not limited to *L. intracellularis* and which polypeptide preferably possesses hemolysin activity.

To improve the immunogenicity of a subject polypeptide of the present invention one or more amino acids not corresponding to the original protein sequence can be added to the amino or carboxyl terminus of the polypeptide. Such extra amino acids are useful for coupling the polypeptide to another peptide or polypeptide, to a large carrier protein or to a solid support. Amino acids that are useful for these purposes include but are not limited to tyrosine, lysine, glutamic acid, aspartic acid, cysteine and derivatives thereof. Additional protein modification techniques can be used such as, e.g., NH_2-acetylation or COOH-terminal amidation, to provide additional means for coupling the polypeptide to another polypeptide, protein, or peptide molecule, or to a solid support. Procedures for coupling polypeptides to each other, or to carrier proteins or solid supports, are well known in the art. Polypeptides containing the above-mentioned extra amino acid residues at either the carboxyl- or amino-termini and either uncoupled or coupled to a carrier or solid support, are consequently within the scope of the present invention.

Furthermore, the polypeptide can be immobilised to a polymeric carrier or support material.

In an alternative embodiment, the immunogenicity of a polypeptide of the present invention may be improved using molecular biology techniques to produce a fusion

protein containing one or more polypeptides of the present invention fused to a carrier molecules such as a highly immunogenic protein. For example, a fusion protein containing a polypeptide of the present invention fused to the highly immunogenic B subunit of cholera toxin can be used to increase the immune response to the polypeptide. The present invention also contemplates fusion proteins comprising a cytokine, such as an interleukin, fused to the subject polypeptide of the present invention, and genes encoding same.

Preferably, the polypeptide of the present invention, or a derivative, homologue or analogue thereof, when administered to a mammal, induces an immune response in said mammal. More preferably, the polypeptide of the present invention, when administered to a mammal, in particular a porcine animal (e.g., a pig) induces a protective immune response against *Lawsonia spp.*, and preferably against *L. intracellularis*, therein. As used herein, the phrase "induction of a protective immune response", and the like, refers to the ability of the administered polypeptide of the present invention to prevent or detectably slow the onset, development, or progression of symptoms associated with *Lawsonia* infection, and preferably, to prevent or detectably slow the onset, development, or progression of symptoms associated with PPE in pigs.

Preferably, the immunogenic polypeptide of the invention comprises an amino acid sequence which is substantially the same as the amino acid sequence set forth in SEQ ID NO: 1 or is at least about 70% identical overall to SEQ ID NO: 1, or is at least about 75% identical to at least about 8 or more contiguous amino acids of SEQ ID NO: 1. In a preferred embodiment, the immunogenic polypeptide of the present invention consists essentially of the amino acid sequence of SEQ D NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence present in pALK12 (ATCC 207195), or about the first fifty amino acids thereof.

For the purposes of nomenclature, the amino acid sequence set forth in SEQ ID NO: 1 represents the hemolysin polypeptide encoded by the *Lawsonia intracellularis tlyA* gene, the nucleotide sequence of which is set forth in SEQ ID NO: 2.

Preferably, the percentage amino acid sequence identity to SEQ ID NO: 1 is at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, and still even more preferably at least about 95% similar to SEQ ID NO: 1.

In determining whether or not two amino acid sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments, differences will arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage sequence identity or similarity between two or more amino acid sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard algorithm known to those skilled in the art. For example, amino acid sequence identities or similarities may be calculated using the GAP programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984). The GAP programme utilizes the algorithm of Needleman and Wunsch (1970) to maximise the number of identical/similar residues and to minimise the number and/or length of sequence gaps in the alignment. Alternatively or in addition, where more than two amino acid sequences are being compared, the ClustalW programme of Thompson *et al* (1994) can be used.

The present invention further encompasses homologues, analogues and derivatives of a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1.

"Homologues" of a polypeptide are those polypeptides which contain amino acid substitutions, deletions and/or additions relative to the polypeptide without altering one or more of its properties, such as its immunogenicity, biological activity or catalytic activity. In such molecules, amino acids can be replaced by other amino acids having similar properties such as, for example, hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α-helical structures or β-sheet structures, and so on.

Substitutional variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues. and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein. Insertions can comprise amino– terminal and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the sequence.

Amino acid variants of the polypeptide of the present invention may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis or other site-directed mutagenesis protocol.

"Analogues" are defined as peptides, oligopeptides and polypeptides which are functionally equivalent to the peptides of the present invention but which contain certain non-naturally occurring or modified amino acid residues as will be known to those skilled in the art. Accordingly, an "analogue" as defined herein need not comprise an amino acid sequence which is similar to the amino acid sequence set

forth herein such as, for example, peptides, oligopeptides and polypeptides which are derived from computational predictions or empirical data revealing the secondary, tertiary or quaternary structure of the hemolysin polypeptide of the present invention, and which therefore do not comprise the same primary amino acid sequence of said hemolysin polypeptide, yet nevertheless mimic or cross-react with B-cell or T-cell epitope of *Lawsonia spp.* and preferably, mimic or cross-react with B-cell or T-cell epitope of *Lawsonia intracellularis*.

For example, mimotopes (polypeptide analogues that cross-react with a B-cell or T-cell epitope of the *Lawsonia* polypeptide of the invention but, however, comprise a different amino acid sequence to said epitope) may be identified by screening random amino acid sequences in peptide libraries with antibodies that bind to a desired T-cell or B-cell epitope. As with techniques for the identification of B-cell or T-cell epitopes as described *supra*, the antibodies used to identify such mimotopes may be polyclonal or monoclonal or recombinant antibodies, in crude or purified form. Mimotopes of a T-cell epitope may then be assayed further for their ability to stimulate T-cell cytotoxic or proliferative responses *in vitro*. Mimotopes are particularly useful as analogues of non-linear (i.e., conformational) epitopes of the polypeptide of the present invention, because conformational epitopes are generally formed from non-contiguous regions in a polypeptide, and the mimotopes provide immunogenic equivalents thereof in the form of a single peptide molecule.

Additionally, the use of polypeptide analogues can result in polypeptides with increased immunogenic and/or antigenic activity, that are less sensitive to enzymatic degradation, and which are more selective. A suitable proline analogue is 2-aminocyclopentane carboxylic acid (βAC^5c) which has been shown to increase the immunogenic activity of a native polypeptide more than 20 times (Mierke *et al*, 1990; Portoghese *et al*, 1990; Goodman *et al*, 1987).

"Derivatives" of a polypeptide described herein are those peptides, oligopeptides and polypeptides which comprise at least about five contiguous amino acid residues of the amino acid sequence set forth in SEQ ID NO: 1. A "derivative" may further comprise

additional naturally-occurring, altered glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence set forth in SEQ ID NO: 1. Alternatively or in addition, a derivative may comprise one or more non-amino acid substituents such as, for example, a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound thereto to facilitate its detection.

Other examples of recombinant or synthetic mutants and derivatives of the peptide immunogens of the present invention include those incorporating single or multiple substitutions, deletions and/or additions therein, such as carbohydrates, lipids and/or proteins or polypeptides. Naturally occurring or altered glycosylated or acylated forms of the subject peptides are particularly contemplated by the present invention. Additionally, homopolymers or heteropolymers comprising one or more copies of the subject peptide listed in SEQ ID NO: 1, or one or more derivatives, homologues or analogues thereof, are within the scope of the invention.

Preferably, homologues, analogues and derivatives of the polypeptide of the invention are "immunogenic", defined hereinafter as the ability of said polypeptide, or a derivative, homologue or analogue thereof, to elicit B cell and/or T cell responses in the host, in response to immunization.

Preferred homologues, analogues and derivatives of the amino acid sequence set forth in SEQ ID NO: 1 include those amino acid variants that function as B cell or T cell epitopes of said amino acid sequence which are capable of mediating an immune response such as, for example, mimotopes of the immunogenic polypeptide described herein which have been produced by synthetic means, such as by Fmoc chemistry. The only requirement of such molecules is that they cross-react immunologically with a polypeptide which comprises the amino acid sequence set forth in SEQ ID NO: 1, or the first 50 amino acid residues thereof, or a derivative thereof which comprises at least 5 contiguous amino acids in length of SEQ ID NO: 1.

As will be apparent to those skilled in the art, such homologues, analogues and

derivatives of the polypeptide of the invention molecules will be useful to prepare antibodies that cross-react with antibodies against said polypeptide and/or to elicit a protective immune response of similar specificity to that elicited by said polypeptide. Such molecules will also be useful in diagnostic and other applications that are immunological in nature such as, for example, diagnostics which utilise one or more immunoassay formats (eg. ELISA, RIA and the like).

Accordingly, the immunogen of the present invention or a derivative, homologue or analogue thereof is useful in vaccine compositions that protect an individual against infection by *L. intracellularis* and/or as an antigen to elicit polyclonal or monoclonal antibody production and/or in the detection of antibodies against *L. intracellularis* in infected animals, particularly in porcine and avian animals.

The present inventors have also shown that the N-terminal region of SEQ ID NO: 1 is particularly unique, as compared to other immunogenic amino acid sequences, including those of the hemolysin molecules of other animal pathogens. Accordingly, peptides, oligopeptides and polypeptides which comprise such unique epitope regions of SEQ ID NO: 1, will have improved specificity compared to other regions of the *Lawsonia spp.* hemolysin molecule. The particular advantages of such peptides will be immediately apparent to those skilled in the production of vaccine compositions, where specificity against a pathogen of interest is an important consideration.

In particular, the present inventors have shown that amino acids 1 to 50 of the *L. intracellularis* hemolysin polypeptide, as set forth in SEQ ID NO: 1, is not highly conserved compared to the corresponding region of other hemolysin polypeptides, being only about 50% identical to the amino acid sequences in the N-terminal region of the *Mycobacterium tuberculosis* and *Serpula hyodysenteriae* hemolysin polypeptides. Accordingly, this region of the *L. intracellularis* hemolysin polypeptide is a promising antigenic peptide for the formulation of *Lawsonia*-specific vaccines and diagnostics for the specific detection of *Lawsonia spp.* in biological samples.

Accordingly, in an alternative embodiment, the present invention provides an isolated

or recombinant immunogenic polypeptide or a derivative, homologue or analogue thereof which comprises, mimics or cross-reacts with a B-cell or T-cell epitope of a *Lawsonia spp.* wherein said polypeptide comprises a sequence of amino acids which has at least about 50% sequence identity to about amino acid residue 1 to about amino acid residue 50 of the *L. intracellularis* hemolysin polypeptide, as set forth in SEQ ID NO: 1. Preferably, the percentage sequence identity to amino acids 1 to 50 of SEQ ID NO: 1 is at least about 60%, more preferably at least about 70%, even more preferably at least about 80% and still even more preferably at least about 90%. In a particularly preferred embodiment, the subject polypeptide will comprise a sequence of amino acids from about amino acid 1 to about amino acid 50 of SEQ ID NO: 1.

A second aspect of the present invention provides a vaccine composition for the prophylaxis or treatment of infection in a mammal or bird by *L. intracellularis* or similar or otherwise related microorganism, said vaccine composition comprising an effective amount of an immunogenic component which comprises an isolated or recombinant polypeptide having at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1 or at least about 50% sequence identity overall to about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO: 1, or an immunogenic homologue, analogue or derivative thereof which is immunologically cross-reactive with *Lawsonia intracellularis;* and one or more carriers, diluents or adjuvants suitable for veterinary or pharmaceutical use.

As used herein, the term "immunogenic component" refers to a peptide, polypeptide or a protein encoded by DNA from, or derived from, *L. intracellularis* or a related microorganism thereto which is capable of inducing a protective immune response in an animal, in particular a porcine or avian animal, whether or not said peptide, polypeptide or protein is in an isolated or recombinant form. Accordingly, the vaccine composition clearly encompasses those vaccine compositions which comprise attenuated, killed or non-pathogenic isolates or forms of *L. intracellularis* or related microorganisms thereto which comprise or express said peptide, polypeptide or protein.

By "protective immune response" is meant that the immunogenic component elicits an immune response in the animal to which the vaccine composition is administered at the humoral and/or cellular level which is sufficient to prevent infection by *Lawsonia intracellularis* or a related microorganism thereto and/or which is sufficient to detectably reduce one or more symptoms or conditions, or to detectably slow the onset of one or more symptoms or conditions, associated with infection by *Lawsonia intracellularis* or a related microorganism thereto in an animal host, as compared to a control infected animal. The term "effective amount" of an immunogenic component present in the vaccine composition refers to that amount of said immunogenic component that is capable of inducing a protective immune response after a single complete dose has been administered, or after several divided doses have been administered.

Preferably, the polypeptide component of the subject vaccine composition comprises an amino acid sequence which is both immunogenic and specific, by virtue of its immunological cross-reactivity with the causative agent of PPE, *Lawsonia intracellularis*. In this regard, it will be apparent from the preceding description that such polypeptide components may comprise an amino acid sequence derived from SEQ ID NO: 1 or a homologue, analogue or derivative of the amino acid sequence set forth in SEQ ID NO: 1 such as, for example, a mimotope of said sequence.

The immunogenic polypeptide or immunogenic homologue, analogue or derivative may be a naturally-occurring peptide, oligopeptide or polypeptide in isolated or recombinant form according to any of the embodiments described *supra* or exemplified herein. Preferably, the immunogenic polypeptide or immunogenic homologue, analogue or derivative is derived from *Lawsonia spp.*, in particular *L. intracellularis* or a microorganism that is related thereto.

Preferably, the immunogenic component has undergone at least one purification step or at least partial concentration from a cell culture comprising *L. intracellularis* or a related microorganism thereto, or from a lysed preparation of *L. intracellularis* cells or related microorganism, or from another culture in which the immunogenic component is recombinantly expressed. The purity of such a component which has the requisite

immunogenic properties is preferably at least about 20% by weight of protein in a particular preparation, more preferably at least about 50%, even more preferably at least about 60%, still more preferably at least about 70% and even more preferably at least about 80% or greater.

The immunogenic component of the vaccine of the present invention can comprise a single peptide, polypeptide or protein, or a range or combination of different peptides, polypeptides or proteins covering different or similar epitopes. In addition or, alternatively, a single polypeptide can be provided with multiple epitopes. The latter type of vaccine is referred to as a polyvalent vaccine. A multiple epitope includes two or more epitopes located within a peptide or polypeptide molecule.

The formulation of vaccines is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pennsylvania, USA.

A particularly useful form of the vaccine is a recombinant vaccine produced, for example, in a vaccine vector, such as but not limited to a cell transfected with a vaccinia virus vector or a bacterial cell capable of expressing the immunogenic component.

The present invention clearly extends to recombinant vaccine compositions in which the immunogenic component at least is contained within killed vaccine vectors prepared, for example, by heat, formalin or other chemical treatment, electric shock or high or low pressure forces. According to this embodiment, the immunogenic component of the vaccine is generally synthesized in a live vaccine vector which is killed prior to administration to an animal.

Furthermore, the vaccine vector expressing the immunogenic component may be non-pathogenic or attenuated. Within the scope of this embodiment are cells that have been transfected with non-pathogenic or attenuated viruses encoding the immunogenic component of the vaccine and non-pathogenic or attenuated cells that

directly express the immunogenic component.

Attenuated or non-pathogenic host cells include those cells which are not harmful to an animal to which the subject vaccine is administered. As will be known to those skilled in the art, "live vaccines" can comprise an attenuated virus vector encoding the immunogenic component or a host cell comprising same, which is capable of replicating in an animal to which it is administered, and using host cell machinery to express the immunogenic component, albeit producing no adverse side-effects therein. Such vaccine vectors may colonise the gut or other organ of the vaccinated animal Such live vaccine vectors are efficacious by virtue of their ability to continually express the immunogenic component in the host animal for a time and at a level sufficient to confer protective immunity against a pathogen which expresses an immunogenic equivalent of said immunogenic component. The present invention clearly encompasses the use of such attenuated or non-pathogenic vectors and live vaccine preparations.

The vaccine vector may be a virus, bacterial cell or a eukaryotic cell such as an avian, porcine or other mammalian cell or a yeast cell or a cell line such as COS, VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK) or MDCK cell lines. Suitable prokaryotic cells include *Mycobacterium spp.*, *Corynebacterium spp.*, *Salmonella spp.*, *Escherichia coli, Bacillus spp.* and *Pseudomonas spp*, amongst others. Bacterial strains which are suitable for the present purpose are well-known in the relevant art (Ausubel *et al*, 1987; Sambrook *et* al, 1989).

Such cells and cell lines are capable of expression of a genetic sequence encoding a peptide, polypeptide or protein of the present invention from *L. intracellularis* in a manner effective to induce a protective immune response in the animal. For example, a non-pathogenic bacterium could be prepared containing a recombinant sequence capable of encoding a peptide, polypeptide or protein from *L. intracellularis*. The recombinant sequence would be in the form of an expression vector under the control of a constitutive or inducible promoter. The bacterium would then be permitted to

colonise suitable locations in a pig's gut and would be permitted to grow and produce the recombinant peptide, polypeptide or protein in amount sufficient to induce a protective immune response against *L. intracellularis*.

In a further alternative embodiment, the vaccine can be a DNA or RNA vaccine comprising a DNA or RNA molecule encoding a peptide, polypeptide or protein of the present invention which is injected into muscular tissue or other suitable tissue in a pig under conditions sufficient to permit transient expression of said DNA or RNA to produce an amount of peptide, polypeptide or protein effective to induce a protective immune response. In a preferred embodiment, the DNA vaccine is in the form of a plasmid, in which the DNA is operably connected with a promoter region capable of expressing the nucleotide sequence encoding the immunogen in cells of the immunized animal.

In the production of a recombinant vaccine, except for a DNA vaccine described herein, it is therefore necessary to express the immunogenic component in a suitable vector system. For the present purpose, the immunogenic component can be expressed by:

(i) placing an isolated nucleic acid molecule in an expressible format, said nucleic acid molecule comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 2 or a protein-encoding homologue, analogue or derivative of SEQ ID NO: 2 selected from the group consisting of:

(a) nucleotide sequences that have at least about 70% sequence identity to SEQ ID NO: 2;

(b) nucleotide sequences that hybridise under at least low stringency hybridisation, preferably under at least moderate stringency conditions, and even more preferably under high stringency conditions, to the complement of SEQ ID NO: 2; and

(c) nucleotide sequences that encode the amino acid sequence set forth in SEQ ID NO: 1 or a homologue, analogue or derivative thereof, including, for example, a mimotope of the amino acid set forth in SEQ ID NO: 1;

(ii) introducing the isolated nucleic acid molecule of (i) in an expressible format into a suitable vaccine vector; and

(iii) incubating or growing the vaccine vector for a time and under conditions sufficient for expression of the immunogenic component encoded by said nucleic acid molecule to occur.

For the purposes of defining the level of stringency, a low stringency is defined herein as being a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C. A moderate stringency is defined herein as being a hybridisation and/or washing carried out in 2xSSC buffer, 0.1% (w/v) SDS at a temperature in the range 45°C to 65°C. A high stringency is defined herein as being a hybridisation and/or wash carried out in 0.1xSSC buffer, 0.1% (w/v) SDS at a temperature of at least 65°C.

Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Those skilled in the art will be aware that the conditions for hybridisation and/or wash may vary depending upon the nature of the hybridisation membrane or the type of hybridisation probe used. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification of the parameters affecting hybridisation between nucleic acid molecules, reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

As used herein, a "nucleic acid molecule in an expressible format" is a protein-encoding region of a nucleic acid molecule placed in operable connection with a promoter or other regulatory sequence capable of regulating expression in the vaccine vector system.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e., upstream activating sequences,

enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. In the present context, the term "promoter" is also used to describe a recombinant, synthetic or fusion molecule, or derivative which confers, activates or enhances the expression of a nucleic acid molecule to which it is operably connected, and which encodes the immunogenic polypeptide. Preferred promoters can contain additional copies of one or more specific regulatory elements to further enhance expression and/or to alter the spatial expression and/or temporal expression of the said nucleic acid molecule.

Placing a nucleic acid molecule under the regulatory control of i.e., "in operable connection with" a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally, but not necessarily, positioned 5′ (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

The prerequisite for producing intact polypeptides in bacteria such as *E. coli* is the use of a strong promoter with an effective ribosome binding site. Typical promoters suitable for expression in bacterial cells such as *E. coli* include, but are not limited to, the *lacz* promoter, temperature-sensitive λ_L or λ_R promoters, T7 promoter or the IPTG-inducible *tac* promoter. A number of other vector systems for expressing the nucleic acid molecule of the invention in *E. coli* are well-known in the art and are described, for example, in Ausubel *et al* (1987) or Sambrook *et al* (1989). Numerous plasmids

with suitable promoter sequences for expression in bacteria and efficient ribosome binding sites have been described, such as for example, pKC30 (λ_L: Shimatake and Rosenberg, 1981), pKK173-3 (*tac*: Amann and Brosius, 1985), pET-3 (T7: Studier and Moffat, 1986), the pFLEX series of expression vectors (Pfizer Inc., CT, USA) or the pQE series of expression vectors (Qiagen, CA), amongst others. Typical promoters suitable for expression in viruses of eukaryotic cells and eukaryotic cells include the SV40 late promoter, SV40 early promoter and cytomegalovirus (CMV) promoter, CMV IE (cytomegalovirus immediate early) promoter amongst others.

Means for introducing the isolated nucleic acid molecule or a genetic construct comprising same into a cell for expression of the immunogenic component of the vaccine composition are well-known to those skilled in the art. The technique used for a given organism depends on the known successful techniques. Means for introducing recombinant DNA into animal cells includes microinjection, transfection mediated by DEAE-dextran, transfection mediated by liposomes such as by using lipofectamine (Gibco, MD, USA) and/or cellfectin (Gibco, MD, USA), PEG-mediated DNA uptake, electroporation and microparticle bombardment such as by using DNA-coated tungsten or gold particles (Agracetus Inc., WI, USA) amongst others.

The immunogenic component of a vaccine composition as contemplated herein exhibits excellent therapeutic activity, for example, in the treatment and/or prophylaxis of PPE when administered in an amount which depends on the particular case. For example, for recombinant peptide molecules, from about 0.5 µg to about 20 mg, may be administered, preferably from about 1 μg to about 10 mg, more preferably from about 10 μg to about 5 mg, and most preferably from about 50 μg to about 1 mg equivalent of the immunogenic component in a volume of about 1ml to about 5 ml. For DNA vaccines, a preferred amount is from about 0.1 μg/ml to about 5 mg/ml in a volume of about 1 to about 5 ml. The DNA can be present in "naked" form or it can be administered together with an agent facilitating cellular uptake (e.g., in liposomes or cationic lipids). The important feature is to administer sufficient immunogen to induce a protective immune response. The above amounts can be administered as stated or calculated per kilogram of body weight. Dosage regime can be adjusted to

provide the optimum therapeutic response. For example, several divided doses can be administered or the dose can be proportionally reduced as indicated by the exigencies of the therapeutic situation. Booster administration may also be required.

The vaccine of the present invention can further comprise one or more additional immunomodulatory components such as, for example, an adjuvant or cytokine molecule, amongst others, that is capable of increasing the immune response against the immunogenic component. Non-limiting examples of adjuvants that can be used in the vaccine of the present invention include the RIBI adjuvant system (Ribi Inc., Hamilton, MT, USA), alum, mineral gels such as aluminium hydroxide gel, oil-in-water emulsions, water-in-oil emulsions such as, for example, Block co-polymer (CytRx, Atlanta GA, USA),QS-21 (Cambridge Biotech Inc., Cambridge MA, USA), SAF-M (Chiron, Emeryville CA, USA), AMPHIGEN® adjuvant, Freund's complete adjuvant; Freund's incomplete adjuvant; and Saponin, QuilA or other saponin fraction, monophosphoryl lipid A, and Avridine lipid-amine adjuvant. Other immunomodulatory agents that can be included in the vaccine include, for example, one or more cytokines, such as interferon and/or interleukin, or other known cytokines. Non-ionic surfactants such as, for example, polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether may also be included in the vaccines of the present invention.

The vaccine composition can be administered in a convenient manner such as by oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or by implantation (e.g., using slow release technology). Depending on the route of administration, the immunogenic component may be required to be coated in a material to protect it from the action of enzymes, acids and other natural conditions which may inactivate it, such as those in the digestive tract.

The vaccine composition may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof, or in oils. Under ordinary conditions of storage and use, these preparations can contain a preservative to prevent the growth of microorganisms. Alternatively, the

vaccine composition can be stored in lyophilised form to be rehydrated with an appropriate vehicle or carrier prior to use.

Pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be fluid to the extent that easy syringeability exists, unless the pharmaceutical form is a solid or semi-solid such as when slow release technology is employed. In any event, it must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms.

The carrier may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents such as, for example,, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents such as, for example,, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption such as, for example,, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter-sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients selected from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any

additional desired ingredient from previously sterile-filtered solution thereof.

The present invention extends to vaccine compositions which confer protection against infection by one or more isolates or sub-types of *L. intracellularis* including those that belong to the same serovar or serogroup as *Lawsonia intracellularis*. The vaccine composition preferably also confers protection against infection by other species of the genus *Lawsonia* or other microorganisms related thereto as determined at the nucleotide, biochemical, structural, physiological and/or immunointeractive level; the only requirement being that said other species or other microorganism expresses a polypeptide which is immunologically cross-reactive to the polypeptide of the invention described herein. For example, such related microorganisms may comprise genomic DNA which is at least about 70% identical overall to the genomic DNA of *Lawsonia intracellularis* as determined using standard genomic DNA hybridisation and analysis techniques.

The terms "serogroup" and "serovar" relate to a classification of microorganisms which is based upon serological typing data, in particular data obtained using agglutination assays such as the microscopic agglutination test (MAT). Those skilled in the art will be aware that serovar and serogroup antigens are a mosaic on the cell surface and, as a consequence there will be no strict delineation between bacteria belonging to a serovar and/or serogroup. Moreover, organisms which belong to different species may be classified into the same serovar or serogroup because they are indistinguishable by antigenic determination. As used herein, the term "serovar" means one or more *Lawsonia* strains which are antigenically-identical with respect to antigenic determinants produced by one or more loci. Quantitatively, serovars may be differentiated from one another by cross-agglutination absorption techniques. As used herein, the term "serogroup" refers to a group of *Lawsonia spp.* whose members cross-agglutinate with shared group antigens and do not cross-agglutinate with the members of other groups and, as a consequence, the members of a serogroup have more or less close antigenic relations with one another by simple cross-agglutination.

The present invention thus clearly extends to vaccine compositions for the treatment and/or prophylaxis of animals, in particular, vaccine compositions for the treatment and/or prophylaxis of porcine and/or avian species, against any bacterium belonging to the same serovar or serogroup as *Lawsonia intracellularis*. Preferably, such organisms will express a polypeptide having an amino acid sequence identity of at least about 70% overall with respect to SEQ ID NO: 1 and/or at least about 50% with respect to amino acids 1 to 50 of SEQ ID NO: 1.

The present invention extends further to vaccine compositions capable of conferring protection against a "genetic variant" of *Lawsonia intracellularis*, the only requirement being that said variant expresses a polypeptide having an overall amino acid sequence identity of at least about 70% with respect to SEQ ID NO: 1 and/or at least about 50% with respect to amino acids 1 to 50 of SEQ ID NO: 1 or a homologue, analogue or derivative thereof which is immunologically cross-reactive thereto. Genetic variants of *L. intracellularis* can be developed by mutation, recombination, conjugation or transformation of *L. intracellularis* or may occur naturally. It will be known to a person skilled in the art how to produce such variants.

In a particularly preferred embodiment, the vaccine composition of the invention is intended for or suitable for the prophylaxis and/or treatment of infection in a porcine or avian animal by *L. intracellularis*.

Those skilled in the art will recognise the general applicability of the invention in vaccinating animals other than porcine and avian animals against *L. intracellularis* and/or related microorganisms. In the general application of the vaccine of the present invention, the only prerequisite is that the animal on which protection is conferred is capable of being infected with *Lawsonia intracellularis* and/or a related microorganism thereto and that, in the case of a related microorganism to *L. intracellularis*, said related microorganism expresses a B-cell or T-cell epitope which mimics or cross-reacts with the polypeptide component of the vaccine composition described herein. Animals which may be protected by the vaccine of the present invention include, but are not limited to, humans, primates, companion animals (e.g., cats, dogs), livestock

animals (e.g., pigs, sheep, cattle, horses, donkeys, goats), laboratory test animals (e.g., mice, rats, guinea pigs, rabbits) and captive wild animals (e.g., kangaroos, foxes, deer) The present invention also extends to the vaccination of birds such as poultry birds, game birds and caged birds.

The present invention further extends to combination vaccines comprising an effective amount of a first immunogenic component comprising the polypeptide of the present invention combined with an effective amount of a second immunogenic component comprising one or more other antigens capable of protecting a porcine animal, or bird, against either *Lawsonia spp.* or another pathogen that infects and causes disease in said animal. In a preferred embodiment, the second immunogenic component is selected from the group consisting of the *L. intracellularis* autolysin, OmpH, FlgE, and SodC polypeptides and homologues, analogues or derivatives thereof, in particular immunogenic variants or derivatives thereof, and nucleic acid molecules encoding same.

The isolated or recombinant polypeptide of the invention or an immunologically-equivalent homologue, analogue or derivative thereof is also useful for the preparation of immunologically interactive molecules which are useful in the diagnosis of infection of an animal by *Lawsonia spp.*, in particular by *L. intracellularis* or a related organism thereto.

As used herein, the term "immunologically interactive molecule" includes antibodies and antibody derivatives and functional equivalents, such as a Fab, or a SCAB (single-chain antibody), any of which optionally can be conjugated to an enzyme, radioactive or fluorescent tag, amongst others. The only requirement of such immunologically interactive molecules is that they are capable of binding specifically to the immunogenic polypeptide of the present invention as hereinbefore described.

Accordingly, a further aspect of the invention extends to an immunologically interactive molecule which is capable of binding to any one or more of the following:

(i) a peptide, oligopeptide or polypeptide which comprises an amino acid

sequence which has at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence having at least about 50% overall sequence identity to amino acid residues 1 to 50 of SEQ ID NO: 1; or

(iii) a homologue, analogue or derivative of (i) or (ii) which mimics a B-cell or T-cell epitope thereof .

In a preferred embodiment, the immunologically interactive molecule is an antibody that binds specifically to a polypeptide consisting of the amino acid of SEQ ID NO: 1, or to the first fifty amino acids thereof.

Conventional methods can be used to prepare the immunologically interactive molecules. For example, by using a polypeptide of the present invention, polyclonal antisera or monoclonal antibodies can be made using standard methods. For example, a mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the polypeptide of the present invention which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a polypeptide include conjugation to carriers or other techniques well known in the art. For example, the polypeptide can be administered in the presence of adjuvant or can be coupled to a carrier molecule, as known in the art, that enhances the immunogenicity of the polypeptide. The progress of immunization can be monitored by detection of antibody titres in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, for example, IgG molecules corresponding to the polyclonal antibodies can be isolated from the antisera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an animal immunised with a peptide of the present invention and fused with myeloma cells by standard somatic cell fusion procedures, thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique originally developed by Kohler and

Milstein (1975), as well as other techniques such as the human B-cell hybridoma technique (Kozbor *et al.*, 1983), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, 1985), and screening of combinatorial antibody libraries (Huse *et al.*, 1989). Hybridoma cells can be isolated and screened immunochemically for production of antibodies that are specifically reactive with the polypeptide, and monoclonal antibodies isolated therefrom.

As with all immunogenic compositions for eliciting antibodies, the immunogenically effective amounts of the peptides of the invention must be determined empirically. Factors to be considered include the immunogenicity of the native peptide, whether or not the peptide will be complexed with or covalently attached to an adjuvant or carrier protein or other carrier, the route of administration for the composition, i.e., intravenous, intramuscular, subcutaneous, *etc.*, and the number of immunizing doses to be administered. Such factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

The term "antibody" as used herein, is intended to include fragments thereof which are also specifically reactive with a peptide that mimics or cross-reacts with a B cell or T cell epitope of the *Lawsonia intracellularis* hemolysin polypeptide set forth in SEQ ID NO: 1. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

It is within the scope of this invention to include any secondary antibodies (monoclonal, polyclonal or fragments of antibodies), including anti-idiotypic antibodies, directed to the first mentioned antibodies discussed above. Both the first and second antibodies can be used in detection assays or a first antibody can be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of a peptide which mimics, or cross-reacts with a B cell or T cell epitope of the *Lawsonia intracellularis* hemolysin polypeptide set forth

in SEQ ID NO: 1 as hereinbefore described.

The antibodies described herein are useful for determining B cell or T cell epitopes of the amino acid sequence set forth in SEQ ID NO: 1 such as, for example, by testing the ability of synthetic peptides to cross-react immunologically with said amino acid sequence or to elicit the production of antibodies which cross-react with said amino acid sequence. Using methods described herein, polyclonal antibodies, monoclonal antibodies or chimeric monoclonal antibodies can also be raised to peptides which mimic or cross-react with a B cell or T cell epitope of the *Lawsonia intracellularis* hemolysin polypeptide set forth in SEQ ID NO: 1.

More particularly, the polyclonal, monoclonal or chimeric monoclonal antibodies can be used to detect the peptides of the invention and/or any homologues, analogues or derivatives thereof, in various biological materials. For example, they can be used in an ELISA, radioimmunoassay, or histochemical test. In other words, the antibodies can be used to test for binding to a polypeptide of the invention or to a homologue, analogue or derivative thereof, in a biological sample to diagnose the presence of *Lawsonia intracellularis* therein.

Accordingly, a further aspect of the invention provides a method of diagnosing infection of an animal by *Lawsonia intracellularis* or a related microorganism thereto, said method comprising the steps of contacting a biological sample derived from said animal with an immunologically interactive molecule which is capable of binding to a peptide, oligopeptide or polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1 or a homologue, analogue or derivative thereof, for a time and under conditions sufficient for an antigen:antibody complex to form, and detecting said complex formation. According to this embodiment of the present invention, the immunologically interactive molecule is preferably an antibody molecule prepared against the *Lawsonia intracellularis* hemolysin polypeptide set forth in SEQ ID NO: 1 or an analogue or derivative thereof.

The biological sample is one which might contain a polypeptide having an amino acid

sequence set forth in SEQ ID NO: 1 or a homologue, analogue or derivative thereof, in particular a biological sample derived from a porcine or avian host of the pathogen *Lawsonia intracellularis* or a related microorganism thereto, and can include any appropriate tissue or fluid sample from the animal. Preferred biological samples are derived from the ileum, caecum, small intestine, large intestine, whole serum or lymph nodes of the porcine or avian host animal being tested. Alternatively or in addition, the biological test sample may comprise faeces or a rectal swab derived from the animal.

Conventional immunoassays can be used to perform this embodiment of the invention. A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target. It will be readily apparent to the skilled technician how to modify or optimise such assays to perform this embodiment of the present invention, and all such modifications and optimisations are encompassed by the present invention.

In one alternative embodiment, the present invention contemplates a method of identifying whether or not an animal has suffered from a past infection, or is currently infected with *Lawsonia intracellularis* or a related microorganism thereto, said method comprising contacting blood or serum derived from said animal with the immunogenic polypeptide of the invention for a time and under conditions sufficient for an antigen:antibody complex to form, and detecting said complex formation. This embodiment differs from the embodiment described *supra* in that it relies upon the detection of circulating antibodies against *Lawsonia intracellularis* or related organism in the animals blood or serum which are present as a consequence of a past or present infection by this pathogen. However, it will be apparent to those skilled in the art that the principle of the assay format is the same. As with other embodiments of the invention referred to *supra*, conventional immunoassays can be used. Persons skilled in the art will readily be capable of varying known immunoassay formats to perform the present embodiment. This embodiment of the invention can also utilise derivatives of

blood and serum which comprise immunologically interactive molecules, for example partially-purified IgG or IgM fractions and buffy coat samples, amongst others. The preparation of such fractions will also be known to those skilled in the art.

A further aspect of the present invention provides an isolated nucleic acid molecule which comprises a sequence of nucleotides which encodes, or is complementary to a nucleic acid molecule which encodes, a peptide, oligopeptide or polypeptide selected from the following:

(i) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which has at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence having at least about 50% sequence identity overall to about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO: 1; or

(iii) a homologue, analogue or derivative of (i) or (ii) which mimics a B-cell or T-cell epitope of *Lawsonia spp.*

In a preferred embodiment, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which encodes, or is complementary to a nucleic acid molecule which encodes, a polypeptide immunogen which comprises, mimics or cross-reacts with a B cell or T cell epitope of the *Lawsonia intracellularis* hemolysin polypeptide set forth in SEQ ID NO: 1.

In a particularly preferred embodiment, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding the *L. intracellularis* hemolysin polypeptide having an amino acid sequence set forth in SEQ ID NO: 1 or encoding about the first fifty amino acid residues thereof.

It is within the scope of the invention to encompass polymeric forms of the immunogenic polypeptide described herein, such as aggregates of the amino acid sequence set forth in SEQ ID NO:1 or a homologue, analogue or derivative thereof or, alternatively, as polypeptides comprising repeats of the amino acid sequence set forth

in SEQ ID NO:1 or a homologue, analogue or derivative thereof. The present invention extends further to nucleic acid molecules encoding such polymeric forms.

Alternatively or in addition, the isolated nucleic acid molecule of the invention further comprises a sequence of nucleotides which has at least about 70% overall sequence identity to the nucleotide sequence set forth in SEQ ID NO: 2 or to a complementary nucleotide sequence thereof. More preferably, the percentage sequence identity to SEQ ID NO: 2 or to a complementary nucleotide sequence thereto is at least about 80%. Still more preferably, the percentage sequence identity is at least about 90%. Yet still more preferably, the percentage sequence identity is at least about 95%.

In a preferred embodiment, the nucleic acid molecule comprises the nucleotide sequence set forth in SEQ ID NO: 2 or the hemolysin-encoding nucleotide sequence present in pALK12 (ATCC 207195), or a degenerate variant thereof, and complements thereof.

In determining whether or not two nucleotide sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments, differences may arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity between two or more nucleotide sequences shall be taken to refer to the number of identical residues between said sequences as determined using any standard algorithm known to those skilled in the art. For example, nucleotide sequences may be aligned and their identity calculated using the BESTFIT programme or other appropriate programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984).

Alternatively or in addition, the isolated nucleic acid molecule of the invention is capable of hybridising under at least low stringency conditions to the nucleotide sequence set forth in SEQ ID NO: 2 or to a complementary nucleotide sequence

thereto or to a nucleic acid fragment comprising at least about 20 contiguous nucleotides in length derived from the sequence set forth in SEQ ID NO: 2 or to a complementary nucleotide sequence thereto.

Preferably, said nucleic acid molecule is capable of hybridising under at least moderate stringency conditions, and even more preferably under high stringency conditions.

The present invention clearly encompasses genetic constructs comprising the subject nucleic acid molecule in an expressible format suitable for the preparation of a recombinant immunogenic polypeptide of the present invention, such as for use in recombinant univalent or polyvalent recombinant vaccines.

In such cases, the nucleic acid molecule will be operably connected to a promoter sequence which can thereby regulate expression of said nucleic acid molecule in a prokaryotic or eukaryotic cell as described *supra*.

The genetic construct optionally further comprises a terminator sequence. The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. A "terminator" is a nucleotide sequence, generally located within the 3′-non-translated region of a gene or mRNA, comprising a polyadenylation signal to facilitate the post-transcriptional addition of a polyadenylate sequence to the 3′-end of a primary mRNA transcript. Terminator sequences may be isolated from the genetic sequences of bacteria, fungi, viruses, animals and/or plants. Terminators active in animal cells are known and described in the literature.

In a preferred embodiment, the genetic construct can be a cloning or expression vector, as known in the art, such as a plasmid, cosmid, or phage, comprising a nucleic acid molecule of the present invention, and host cells transformed or transfected therewith. In a non-limiting embodiment, the vector is plasmid pALK12 (ATCC Accession No. 207195).

The genetic constructs of the present invention are particularly useful for producing the

immunogenic component of the vaccine composition described herein or for use in a DNA vaccine.

A range of genetic diagnostic assays to detect infection of an animal by *Lawsonia intracellularis* or a related microorganism can be employed using the nucleic acid molecule described herein such as, for example, assays based upon the polymerase chain reaction (PCR) and nucleic acid hybridisation. All such assays are contemplated in the present invention.

Accordingly, a still further aspect of the invention provides a diagnostic method of detecting *Lawsonia intracellularis* or related microorganism in a biological sample derived from an animal subject, said method comprising the steps of hybridising one or more probes or primers derived from the nucleotide sequence set forth in SEQ ID NO: 2 or a complementary nucleotide sequence thereto or a homologue, analogue or derivative thereof, to a DNA or RNA molecule present in said sample and then detecting said hybridisation using a detection means.

As used herein, the term "probe" refers to a nucleic acid molecule which is derived from the nucleotide sequence set forth in SEQ ID NO: 2 and which is capable of being used in the detection thereof. Probes may comprise DNA (single-stranded or double-stranded) or RNA (i.e., riboprobes) or analogues thereof.

The term "primer" refers to a probe as hereinbefore defined which is further capable of being used to amplify a nucleotide sequence from *Lawsonia intracellularis* or a related microorganism thereto in a PCR.

Preferred probes and primers include fragments of the nucleotide sequence set forth in SEQ ID NO: 2 and synthetic single-stranded DNA or RNA molecules of at least about 15 nucleotides in length derived from the sequence set forth in SEQ ID NO: 2 or a complementary nucleotide sequence thereto.

Preferably, probes and primers according to this embodiment will comprise at least

about 20 contiguous nucleotides derived from SEQ ID NO: 2 or a complementary sequence thereto, even more preferably at least about 25 contiguous nucleotides, still even more preferably at least about 50 contiguous nucleotides and even more preferably at least about 100 nucleotides to about 500 nucleotides derived from the sequence set forth in SEQ ID NO: 2 or a complement thereof. Probes and primers comprising the full-length of SEQ ID NO: 2 or a complementary nucleotide sequence thereto are also encompassed by the present invention.

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which encodes a polypeptide that is functionally equivalent to the polypeptide encoded by the nucleic acid molecule of the present invention or to a polypeptide which is a homologue, analogue or derivative of SEQ ID NO: 1, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

"Analogues" of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which encodes a functionally-equivalent polypeptide to the polypeptide encoded by the nucleic acid molecule of the present invention or a homologue, analogue or derivative of a polypeptide having the amino acid sequence of SEQ ID NO: 1, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule such as, for example, carbohydrates, radiochemicals including radio nucleotides, reporter molecules such as, but not limited to biotin, DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

"Derivatives" of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains at least about 50% nucleotide sequence identity to 15 or more contiguous nucleotides present in the nucleotide sequence set forth in SEQ ID NO: 2 or a complementary nucleotide sequence thereto. Generally, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention

include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional nucleotide sequence variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide sequence variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place. In a preferred embodiment, such substitutions are selected based on the degeneracy of the genetic code, as known in the art, with the resulting substitutional variant encoding the amino acid sequence of SEQ ID NO: 1 or at least about the first 50 amino acids thereof.

Probes or primers can comprise inosine, adenine, guanine, thymidine, cytidine or uracil residues or functional analogues or derivatives thereof that are capable of being incorporated into a polynucleotide molecule, provided that the resulting probe or primer is capable of hybridising under at least low stringency conditions to SEQ ID NO: 2 or to a complementary nucleotide sequence thereof, or is at least about 70% identical to SEQ ID NO: 2 or to a complementary nucleotide sequence thereof.

The biological sample according to this aspect of the invention includes any organ, tissue, cell or exudate which contains or is likely to contain *Lawsonia intracellularis* or a nucleic acid derived therefrom. A biological sample can be prepared in a suitable solution such as, for example, an extraction buffer or suspension buffer. The present invention extends to the testing of biological solutions thus prepared, the only requirement being that said solution at least comprises a biological sample as described herein.

The diagnostic assay of the present invention is useful for the detection of *Lawsonia intracellularis* or a microorganism which is related thereto which expresses the hemolysin polypeptide of the present invention or a hemolysin-like polypeptide.

The present invention clearly contemplates diagnostic assays which are capable of both genus-specific and species-specific detection. Accordingly, in one embodiment, the probe or primer, or a homologue, analogue or derivative thereof, comprises DNA capable of being used to detect multiple *Lawsonia spp.* In an alternative embodiment, the probe or primer or a homologue, analogue or derivative thereof comprises DNA capable of being used to distinguish *Lawsonia intracellularis* from related microorganisms.

Less-highly conserved regions within SEQ ID NO: 2, such as those encoding about amino acid residues 1 to 50 of the *Lawsonia intracellularis* hemolysin polypeptide are particularly useful as species-specific probes and/or primers for the detection of *L. intracellularis* and very closely related species.

Furthermore, the diagnostic assays described herein can be adapted to a genus-specific or species-specific assay by varying the stringency of the hybridisation step. Accordingly, a low stringency hybridisation can be used to detect several different species of *Lawsonia* in one or more biological samples being assayed, while a high stringency hybridisation can be used to distinguish *Lawsonia intracellularis* from such other species.

The detection means according to this aspect of the invention may be any nucleic acid-based detection means such as, for example, nucleic acid hybridisation techniques or paper chromatography hybridisation assay (PACHA), or an amplification reaction such as PCR, or nucleic acid sequence-based amplification (NASBA) system. The invention further encompasses the use of different assay formats of said nucleic acid-based detection means, including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), single-strand chain polymorphism (SSCP), amplification and mismatch detection (AMD), interspersed repetitive sequence polymerase chain reaction (IRS-PCR), inverse polymerase chain reaction (iPCR), *in situ* polymerase chain reaction and reverse transcription polymerase chain reaction (RT-PCR), amongst others.

Where the detection means is a nucleic acid hybridisation technique, the probe can be labelled with a reporter molecule capable of producing an identifiable signal (e.g., a radioisotope such as ^{32}P or ^{35}S, or a biotinylated molecule). According to this embodiment, those skilled in the art will be aware that the detection of said reporter molecule provides for identification of the probe and that, following the hybridisation reaction, the detection of the corresponding nucleotide sequences in the biological sample is facilitated. Additional probes can be used to confirm the assay results obtained using a single probe.

A variation of the nucleic acid hybridisation technique contemplated by the present invention is the paper chromatography hybridisation assay (PACHA) described by Reinhartz *et al.* (1993) and equivalents thereof, wherein a target nucleic acid molecule is labelled with a reporter molecule such as biotin, applied to one end of a nitrocellulose or nylon membrane filter strip and subjected to chromatography under the action of capillary or other forces (e.g., an electric field) for a time and under conditions sufficient to promote migration of said target nucleic acid along the length of said membrane to a zone at which a DNA probe is immobilised thereto such as, for example, in the middle region. According to this detection format, labelled target nucleic acid comprising the *Lawsonia spp.* nucleotide sequences complementary to the probe will hybridise thereto and become immobilised in that region of the membrane to which the probe is bound. Non-complementary sequences to the probe will diffuse past the site at which the probe is bound. The target nucleic acid may comprise a crude or partially-pure extract of DNA or RNA or, alternatively, an amplified or purified DNA. Additional variations of this detection means which utilise the nucleotide sequences described herein are clearly encompassed by the present invention.

Wherein the detection means is a RFLP, nucleic acid derived from the biological sample, in particular DNA, is digested with one or more restriction endonuclease enzymes and the digested DNA is subjected to electrophoresis, transferred to a solid support such as, for example, a nylon or nitrocellulose membrane, and hybridised to a probe optionally labelled with a reporter molecule as hereinbefore defined.

According to this embodiment, a specific pattern of DNA fragments is displayed on the support, wherein said pattern is preferably specific for a particular *Lawsonia species.*, to enable the user to distinguish between different species of the bacterium.

Wherein the detection means is an amplification reaction such as, for example, a polymerase chain reaction or a nucleic acid sequence-based amplification (NASBA) system or a variant thereof, one or more nucleic acid primer molecules of at least 15 contiguous nucleotides in length derivable from SEQ ID NO: 2 or its complementary nucleotide sequence, or a homologue, analogue or derivative thereof, is hybridised to nucleic acid derived from a biological sample, and nucleic acid copies of the hemolysin-encoding genetic sequences in said sample, or a part or fragment thereof, are enzymically-amplified.

Those skilled in the art will be aware that there must be a sufficiently high percentage of nucleotide sequence identity between the primers and the sequences in the biological sample template molecule to which they hybridise (i.e., the "template molecule"). As stated previously, the stringency conditions can be selected to promote hybridisation.

Preferably, each primer is at least about 95% identical to a region of SEQ ID NO: 2 or its complementary nucleotide sequence in the template molecule to which it hybridises.

Those skilled in the art will also be aware that, in one format, PCR provides for the hybridisation of non-complementary primers to different strands of the template molecule, such that the hybridised primers are positioned to facilitate the 5′– 3′ synthesis of nucleic acid in the intervening region, under the control of a thermostable DNA polymerase enzyme. As a consequence, PCR provides an advantage over other detection means in so far as the nucleotide sequence in the region between the hybridised primers may be unknown and unrelated to any known nucleotide sequence.

In an alternative embodiment, wherein the detection means is AFLP, the primers are selected such that, when nucleic acid derived from the biological sample, in particular

DNA, is amplified, different length amplification products are produced from different *Lawsonia spp.* The amplification products can be subjected to electrophoresis, transferred to a solid support such as, for example, a nylon or nitrocellulose membrane, and hybridised to a probe optionally labelled with a reporter molecule as hereinbefore described. According to this embodiment, a specific pattern of amplified DNA fragments is displayed on the support, said pattern optionally specific for a particular *Lawsonia ssp.*, to enable the user to distinguish between different species of the bacterium in much the same way as for RFLP analysis.

The technique of AMD facilitates, not only the detection of *Lawsonia spp.* DNA in a biological sample, but also the determination of nucleotide sequence variants which differ from the primers and probes used in the assay format. Wherein the detection means is AMD, the probe is end-labelled with a suitable reporter molecule and mixed with an excess of the amplified template molecule. The mixtures are subsequently denatured and allowed to renature to form nucleic acid "probe:template hybrid molecules" or "hybrids", such that any nucleotide sequence variation between the probe and the temple molecule to which it is hybridised will disrupt base-pairing in the hybrids. These regions of mismatch are sensitive to specific chemical modification using hydroxylamine (mismatched cytosine residues) or osmium tetroxide (mismatched thymidine residues), allowing subsequent cleavage of the modified site using piperidine. The cleaved nucleic acid may be analysed using denaturing polyacrylamide gel electrophoresis, followed by standard nucleic acid hybridisation as described *supra,* to detect the *Lawsonia*-derived nucleotide sequences. Those skilled in the art will be aware of the means of end-labelling a genetic probe according to the performance of the invention described in this embodiment.

According to this embodiment, the use of a single end-labelled probe allows unequivocal localisation of the sequence variation. The distance between the point(s) of sequence variation and the end-label is represented by the size of the cleavage product.

In an alternative embodiment of AMD, the probe is labelled at both ends with a reporter

molecule, to facilitate the simultaneous analysis of both DNA strands.

Wherein the detection means is RT-PCR, the nucleic acid sample comprises an RNA molecule which is a transcription product of *Lawsonia*-derived DNA or a homologue, analogue or derivative thereof. As a consequence, this assay format is particularly useful when it is desirable to determine expression of one or more *Lawsonia* genes. According to this embodiment, the RNA sample is reverse-transcribed to produce the complementary single-stranded DNA which is subsequently amplified using standard procedures.

Variations of the embodiments described herein are described in detail by McPherson *et al.* (1991).

The present invention clearly extends to the use of any and all detection means referred to *supra* for the purposes of diagnosing *Lawsonia spp.* and in particular *Lawsonia intracellularis* infection in animal.

The amplification reaction detection means described *supra* can be further coupled to a classical hybridisation reaction detection means to further enhance sensitivity and specificity of the inventive method, such as by hybridising the amplified DNA with a probe which is different from any of the primers used in the amplification reaction.

Similarly, the hybridisation reaction detection means described *supra* can be further coupled to a second hybridisation step employing a probe which is different from the probe used in the first hybridisation reaction.

A further aspect of the invention provides an isolated probe or primer derived from SEQ ID NO: 2 or a complementary nucleotide sequence thereto.

The present invention is further described by the following non-limiting examples.

EXAMPLE 1
SOURCES OF PIG TISSUE

Infected Pig Intestines

Sections of grossly thickened ilea were taken from pigs naturally or experimentally affected by PPE. The presence of *L. intracellularis* bacteria in the ilea was confirmed using immunofluorescent staining with specific monoclonal antibodies (McOrist *et al*, 1987). An example of a suitable antibody is monoclonal antibody IG4 available from the University of Edinburgh, UK.

EXAMPLE 2
ISOLATION OF *LAWSONIA INTRACELLULARIS* BACTERIA FROM THE INFECTED PIG ILEUM

Lawsonia intracellularis bacteria were extracted directly from lesions of PPE in pigs by filtration and further purified over a Percoll (Pharmacia, Uppsala, Sweden) gradient as follows. Infected ilea were collected from pigs and the presence of *L. intracellularis* was confirmed histologically before storage at -80°C. Sections of ileum were thawed and approximately 8g of infected mucosa were scraped from the intestinal wall. The mucosa was homogenised with 40 ml sterile phosphate buffered saline (PBS) on half speed for 10 seconds using a Sorvall omnimixer. This suspension was centrifuged at 2000 *xg* for 4 minutes. The supernatant was discarded and the cell pellet was resuspended in 40 ml PBS and re-centrifuged. This washing step was repeated twice. The cell pellet was then resuspended in 20 ml PBS and homogenised at full speed for one minute to release *L. intracellularis* bacteria.

This homogenate was centrifuged at 1000 *xg* for 4 minutes giving a pellet containing a crude mixture of homogenised epithelial cells and intestinal bacteria. The supernatant was filtered using filters with pore sized 3 μm, 1.2 μm and 0.8 μm (Millipore Corporation, MA, USA). The filtrate was centrifuged at 8000 *xg* for 30 minutes, resulting in a small pellet of *L. intracellularis* bacteria. The *L. intracellularis*

bacteria were further purified using a 45% self forming percoll gradient as follows: 2 mls of the bacterial preparation was mixed by inversion into 30 mls of a 45% self forming Percoll (Pharmacia LKB, Uppsala, Sweden) gradient (45% v/v of Percoll, 150 mM NaCl). The gradients were centrifuged in a Sorvall centrifuge using the SS34 rotor, at 20,000 rpm for 30 minutes at 4°C. Usually a number of bands form within the gradient. The band (usually located approx. 10-20 mm from the base of the tube) containing the *L. intracellularis* bacteria was collected and the volume made up to 16 mls with PBS. The solution was then centrifuged for 15 minutes at 8000 rpm. The resultant pellet was washed with PBS before being resuspended in a final volume of approximately one ml.

EXAMPLE 3

PURIFICATION OF *LAWSONIA INTRACELLULARIS* GENOMIC DNA

Genomic DNA was extracted from percoll-gradient purified *Lawsonia intracellularis* bacteria recovered from infected pig ilea scrapings (Example 2) by the methods described by Anderson *et al* (1984) and Sambrook *et al* (1989).

Briefly, the *L. intracellularis* cells were pelleted by centrifugation at 14,000 x g at 4°C for 15 min. The cells were resuspended in 10 ml of TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and centrifuged as before. The pellet was then resuspended in 4 ml of TE buffer containing 4 mg/ml lysozyme (Sigma Chemical Co.) and incubated at 37°C for 20 min. SDS and proteinase K (Promega, WI, USA) were added to final concentrations of 1% (w/v) and 200 μg/ml, respectively, and incubation was continued at 45°C for 4 hours. The lysate was then extracted with an equal volume of phenol, phenol:chloroform (1:1) and chloroform, respectively, and the nucleic acids were recovered from the supernatant by ethanol precipitation. The pellet was gently dissolved in TE, treated with RnaseA (Promega, WI, USA) at 37°C for 30 min and then digested with proteinase K in the presence of 0.5% (w/v) SDS for 1 h at 50°C. After another round of phenol:chloroform (1:1) and ethanol precipitation, the purified DNA was dissolved in TE. The DNA was then stored at 4°C.

EXAMPLE 4

IMMUNOSCREENING OF A *L. INTRACELLULARIS* LIBRARY USING EXPERIMENTAL SERA FROM VACCINATED PIGS

The genomic DNA from Example 3 was partially digested with the restriction endonuclease *Sau*3A (Promega) and ligated into Lambda ZAP Express (Stratagene, CA, USA). The lambda library was plated on a lawn of *E. coli* XLI-Blue cells at a density of 1,000 phage forming units (pfu) per 150 mm L-broth agar plate. The library was screened using the method described in the Protoblot Technical Manual (Promega, WI, USA). The filters were blocked in blocking buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 and 5% blotto,) prior to screening with sera from the pigs Y12 and/or 395. The pigs Y12 and 395 had previously been immunised with formalin-killed *L. intracellularis* and heat-killed *L. intracellularis*, respectively, as described in International Patent Application No. PCT/AU96/00767. Positive plaques identified in the primary screen were picked, replated at a lower density and rescreened with either or both sera until an individual positive plaque was identified. Plasmid DNA from the positive lambda phage clone was isolated by *in vivo* excision, as recommended by the manufacturer (Stratagene, CA, USA). This clone contained the partial *tlyA* gene of *L. intracellularis*.

EXAMPLE 5

ANALYSIS OF *L. INTRACELLULARIS* EXPRESSING PHAGE CLONES

Phagemid DNA from positive λZAP Express phage clones was isolated by *in vivo* excision, by the conditions recommended by the manufacturer (Stratagene).

Plasmid DNA for restriction analysis was extracted by alkaline-lysis, as described by Sambrook *et al* (1989), and for automated sequencing, using the High Pure Plasmid Kit, as recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany).

The nucleotide sequence (320 bp) of the 3' region of the *tlyA* gene of *Lawsonia*

intracelluaris was obtained from the *lambda* clone described in Example 4. The remaining portion of the *tlyA* gene was amplified from *L. intracellularis* genomic DNA, using the Vectorette System as described by the manufacturers (Genosys Biotechnologies Inc., TX, USA).

Briefly, the Vectorette System enables the amplification of specific DNA fragments where the sequence of only one primer is known. There are three basic steps as follows:

(i) digestion of target DNA (*L. intracelluaris* genomic DNA in this case) with a restriction enzyme;

(ii) ligation of synthetic Vectorette oligonucleotides to the digested DNA; and

(iii) amplification of the remaining nucleotide sequence of the *tlyA* gene using a specific primer to the known sequence of *tlyA* obtained from the *lambda* clone described in Example 4 and a second primer directed toward the ligated synthetic Vectorette oligonucleotide.

The nucleotide sequence of the amplified product containing the *tlyA* gene was then obtained.

DNA sequencing of amplified DNA and isolated clone inserts was performed by the Dye-terminator method of automated sequencing (ABI Biosystems, CA, USA).

The nucleotide sequence of the complete coding region of the *tlyA* gene is set out in SEQ ID NO: 2.

EXAMPLE 6

IDENTIFICATION OF *L. INTRACELLULARIS* COMPONENTS

Sequence similarity of the DNA molecules encoding putative vaccine candidates identified from Example 4 and 5, was identified using BLAST algorithms (Gish and States, 1993) to search GenBANK. The nucleotide sequence set forth herein as SEQ

ID NO: 2 and its corresponding deduced amino acid sequence set forth as SEQ ID NO: 1 have some degree of sequence similarity to hemolysin-encoding genes and polypeptides respectively, that are derived from other pathogenic microorganisms (Figure 1).

Unique regions of the *Lawsonia intracellularis* hemolysin polypeptide are apparent from a comparison of the amino acid sequence of this polypeptide to those from other microorganisms (Figure 1).

EXAMPLE 7
PREPARATION OF BIOLOGICAL MATERIAL FOR DEPOSIT - AMPLIFICATION OF *tlyA*

Template DNA was purified from pig intestinal mucosa isolated from the ileum of pigs infected with *L. intracellularis*. DNA purification from intestinal mucosa was performed according to the method of Nollau *et al.* (1996). Due to the presence of contaminating polysaccharides and other material, the DNA content of the samples was not quantified and samples were used empirically in PCR amplifications.

The PCR amplifications consisted of 1 μl DNA isolated from infected pig intestinal mucosa, 1 μM each of the forward (RA168: 5′ AAATAATAAGATGAG 3′; SEQ ID NO:3) and reverse primers (RA169: 5′ ATAGAATACAAATTATAATAAG 3′; SEQ ID NO:4), 7.5 units KlenTaqI polymerase (Ab Peptides, Inc., St. Louis, Missouri), 0.075 units *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, California), 1x PC2 (KlenTaqI) buffer and 0.2 mM dNTPs in a 50 μl volume. PCR was carried out in 4 stages: (i) 94°C for 5 min; (ii) 94°C for 1 min, 58°C for 30 seconds, 72°C for 2 min, x 33 cycles; (iii) 72°C for 10 min, (iv) hold at 4°C.

The PCR fragment comprising the *tlyA* gene of *L. intracellularis* was subcloned into pCR2.1-TOPO (Invitrogen Corp., Carlsbad, CA) and designated pALK12.

DEPOSIT OF BIOLOGICAL MATERIAL

The plasmid pALK12 was deposited with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, VA 20110, USA on 8th April, 1999 and was assigned ATCC Accession No. 207195.

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WE CLAIM:

1. An isolated or recombinant immunogenic polypeptide which comprises, mimics or cross-reacts with a B-cell or T-cell epitope of a *Lawsonia spp.* hemolysin polypeptide.

2. The isolated or recombinant immunogenic polypeptide of claim 1 capable of eliciting the production of antibodies against *Lawsonia spp.* when administered to an avian or porcine animal.

3. The isolated or recombinant immunogenic polypeptide of claim 1 capable of conferring a protective immune response against *Lawsonia spp.* when administered to an avian or porcine animal.

4. The isolated or recombinant immunogenic polypeptide of claim 2 wherein the *Lawsonia spp.* is *L. intracellularis.*

5. The isolated or recombinant immunogenic polypeptide of claim 3 wherein the *Lawsonia spp.* is *L. intracellularis.*

6. An isolated or recombinant immunogenic polypeptide selected from the following:

(i) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which has at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence having at least about 50% sequence identity overall to amino acid residues 1 to 50 of SEQ ID NO: 1; or

(iii) a homologue, analogue or derivative of (i) or (ii) which mimics a B-cell or T-cell epitope of a *Lawsonia spp.* hemolysin polypeptide.

7. The isolated or recombinant immunogenic polypeptide of claim 6 capable of eliciting the production of antibodies against *Lawsonia spp.* in a porcine or avian

animal.

8. The isolated or recombinant immunogenic polypeptide of claim 7 capable of conferring a protective immune response against *Lawsonia spp.* in a porcine or avian animal.

9. The isolated or recombinant immunogenic polypeptide of claim 8, capable of inducing humoral immunity against *Lawsonia spp.* in a porcine or avian animal.

10. The isolated or recombinant immunogenic polypeptide of claim 9, capable of inducing humoral immunity against *Lawsonia spp.* in a porcine animal.

11. The isolated or recombinant immunogenic polypeptide of claim 8 wherein the *Lawsonia spp.* is *L. intracellularis.*

12. The isolated or recombinant immunogenic polypeptide of claim 10 wherein the *Lawsonia spp.* is *L. intracellularis.*

13. The isolated or recombinant immunogenic polypeptide of claim 6 that comprises the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195) and is capable of eliciting the production of antibodies against *Lawsonia intracellularis* when administered to an avian or porcine animal.

14. The isolated or recombinant immunogenic polypeptide of claim 13 that consists essentially of the amino acid sequence of SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195).

15. The isolated or recombinant immunogenic polypeptide of claim 13 or 14 capable of inducing a protective immune response against *Lawsonia intracellularis* in a porcine or avian animal.

16. The isolated or recombinant immunogenic polypeptide of claim 15 capable of inducing a protective immune response against *Lawsonia intracellularis* in a porcine animal.

17. The isolated or recombinant immunogenic polypeptide of claim 6 that comprises amino acid residues about 1 to about 50 of SEQ ID NO: 1 and is capable of eliciting the production of antibodies against *Lawsonia intracellularis* when administered to an avian or porcine animal.

18. The isolated or recombinant immunogenic polypeptide of claim 17 that consists essentially of about amino acid 1 to about amino acid 50 of SEQ ID NO: 1.

19. The isolated or recombinant immunogenic polypeptide of claim 17 capable of inducing a protective immune response against *Lawsonia intracellularis* in a porcine or avian animal.

20. The isolated or recombinant immunogenic polypeptide of claim 19 capable of inducing a protective immune response against *Lawsonia intracellularis* in a porcine animal.

21. A vaccine composition for the prophylaxis or treatment of infection of an animal by *Lawsonia spp.*, said vaccine composition comprising an effective amount of an immunogenic component which comprises an isolated or recombinant polypeptide having at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1 or at least about 50% sequence identity overall to amino acid residues 1 to 50 of SEQ ID NO: 1 or an immunogenic homologue, analogue or derivative thereof which is immunologically cross-reactive with *Lawsonia intracellularis;* and one or more carriers, diluents or adjuvants suitable for veterinary or pharmaceutical use.

22. The vaccine composition according to claim 21 wherein the *Lawsonia spp.* is *L. intracellularis*.

23. The vaccine composition according to claim 22 wherein the immunogenic component comprises an isolated or recombinant polypeptide that comprises the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195).

24. The vaccine composition of claim 23, wherein the immunogenic component consists essentially of the amino acid sequence of SEQ ID NO: 1.

25. The vaccine composition according to claim 22 wherein the immunogenic component comprises an isolated or recombinant polypeptide that comprises about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO: 1.

26. The vaccine composition of claim 25, wherein the immunogenic component consists essentially of about amino acid 1 to about amino acid 50 of SEQ ID NO: 1.

27. A combination vaccine composition for the prophylaxis or treatment of infection of an animal by *Lawsonia spp.*, said vaccine composition comprising:

(i) a first immunogenic component which comprises an isolated or recombinant polypeptide having at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1 or at least about 50% sequence identity overall to amino acid residues 1 to 50 of SEQ ID NO: 1 or an immunogenic homologue, analogue or derivative thereof which is immunologically cross-reactive with *Lawsonia intracellularis;*

(ii) a second immunogenic component comprising an antigenic *L. intracellularis* peptide, polypeptide or protein; and

(iii) one or more carriers, diluents or adjuvants suitable for veterinary or pharmaceutical use.

28. A vaccine vector that comprises, in an expressible form, an isolated nucleic acid molecule having a nucleotide sequence that encodes an isolated or recombinant immunogenic polypeptide which comprises the amino acid sequence set forth in SEQ ID NO: 1, such that said immunogenic polypeptide is expressible at a level sufficient

to confer immunity against *Lawsonia spp.*, when administered to a porcine or avian animal.

29. The vaccine vector of claim 28 wherein the immunogenic polypeptide is expressed using the steps of:

(i) placing an isolated nucleic acid molecule which comprises the nucleotide sequence set forth in SEQ ID NO: 2 or degenerate variant, a homologue, analogue or derivative thereof which has at least about 70% sequence identity thereto, in operable connection with a promoter sequence;

(ii) introducing the isolated nucleic acid molecule and promoter sequence of step (a) into the vaccine vector; and

(iii) incubating, growing, or propagating the vaccine vector for a time and under conditions sufficient for expression of the immunogenic polypeptide encoded by said nucleic acid molecule to occur.

30. The vaccine vector of claim 28 wherein the *Lawsonia spp.* is *L. intracellularis*.

31. A polyclonal or monoclonal antibody molecule that is capable of binding specifically to a hemolysin polypeptide or a derivative of a hemolysin polypeptide that is derived from *Lawsonia spp.* and has at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1.

32. The antibody molecule of claim 31 wherein the hemolysin polypeptide or derivative thereof comprises the amino acid sequence set forth in SEQ ID NO: 1.

33. The antibody molecule of claim 31 wherein the hemolysin polypeptide or derivative thereof comprises about amino acid 1 to about amino acid 50 of SEQ ID NO: 1.

34. A method of diagnosing infection of a porcine or avian animal by *Lawsonia intracellularis* or a microorganism that is immunologically cross-reactive thereto, said method comprising the steps of contacting a biological sample derived from said

animal with the antibody molecule of claim 31 for a time and under conditions sufficient for an antigen:antibody complex to form, and then detecting said complex formation.

35. The method of claim 34 wherein the biological sample comprises whole serum, lymph nodes, ileum, caecum, small intestine, large intestine, faeces or a rectal swab derived from a porcine animal.

36. A method of identifying whether or not a porcine or avian animal has suffered from a past infection, or is currently infected, with *Lawsonia intracellularis* or a microorganism that is immunologically cross-reactive thereto, said method comprising contacting blood or serum derived from said animal with the immunogenic polypeptide of claim 1 for a time and under conditions sufficient for an antigen:antibody complex to form and then detecting said complex formation.

37. An isolated nucleic acid molecule which comprises a sequence of nucleotides that encodes, or is complementary to a nucleic acid molecule that encodes, a peptide, oligopeptide or polypeptide selected from the following:

(i) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which has at least about 70% sequence identity overall to the amino acid sequence set forth in SEQ ID NO: 1;

(ii) a peptide, oligopeptide or polypeptide which comprises an amino acid sequence which comprises an amino having at least about 50% sequence identity overall to amino acid residue 1 to about amino acid residue 50 of SEQ ID NO: 1; or

(iii) a homologue, analogue or derivative of (i) or (ii) which mimics a B-cell or T-cell epitope of *Lawsonia spp*.

38. The isolated nucleic acid molecule of claim 37, wherein the peptide, oligopeptide or polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 1 or the amino acid sequence encoded by the hemolysin-encoding nucleotide sequence of pALK12 (ATCC 207195) or about amino acid residue 1 to about amino acid residue 50 thereof, or a B-cell epitope or T-cell epitope thereof.

39. The isolated nucleic acid molecule of claim 38 comprising the nucleotide sequence set forth in SEQ ID NO: 2 or a complementary nucleotide sequence thereto, or a degenerate variant thereof.

40. The isolated nucleic acid molecule of claim 39 consisting essentially of the nucleotide sequence of SEQ ID NO: 2 or a degenerate variant thereof.

41. The isolated nucleic acid molecule of claim 38 comprising that portion of the nucleotide sequence of SEQ ID NO. 2, or a degenerate variant thereof, which encodes about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO: 2.

42. The isolated nucleic acid molecule of claim 41 consisting essentially of that portion of the nucleotide sequence of SEQ ID NO: 2, or a degenerate variant thereof, which encodes about amino acid residue 1 to about amino acid residue 50 of SEQ ID NO: 2

43. A method of detecting *Lawsonia intracellularis* or related microorganism in a biological sample derived from a porcine or avian animal subject, said method comprising the steps of hybridising one or more probes or primers derived from the nucleotide sequence set forth in SEQ ID NO: 2 or a complementary nucleotide sequence thereto to said sample and then detecting said hybridisation using a detection means.

44. The method of claim 43 wherein the biological sample comprises whole serum, lymph nodes, ileum, caecum, small intestine, large intestine, faeces or a rectal swab derived from a porcine animal.

45. The method of claim 44 wherein the detection means comprises any nucleic acid based hybridisation or amplification reaction.

46. A probe or primer having at least about 15 contiguous nucleotides in length derived from SEQ ID NO: 2 or a complementary nucleotide sequence thereto.

47. A plasmid designated pALK12 (ATCC Accession No. 207195).

48. The combination vaccine composition according to claim 27, wherein the second immunogenic component comprises an antigenic *L. intracellularis* peptide, polypeptide or protein selected from the group consisting of SodC, OmpH, FlgE and autolysin.

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(57) Abstract

The present invention relates generally to therapeutic compositions for the treatment and/or prophylaxis of intestinal disease conditions in animals and birds caused or exacerbated by *Lawsonia intracellularis* or similar or otherwise related microorganism. In particular, the present invention provides a novel gene derived from *Lawsonia intracellularis* which encodes an immunogenic hemolysin peptide, polypeptide or protein that is particularly useful as an antigen in vaccine preparation for conferring humoral immunity against *Lawsonia intracellularis* and related pathogens in animal hosts. The present invention is also directed to methods for the treatment and/or prophylaxis of such intestinal disease conditions and to diagnostic agents and procedures for detecting *Lawsonia intracellularis* or similar or otherwise related microorganisms.

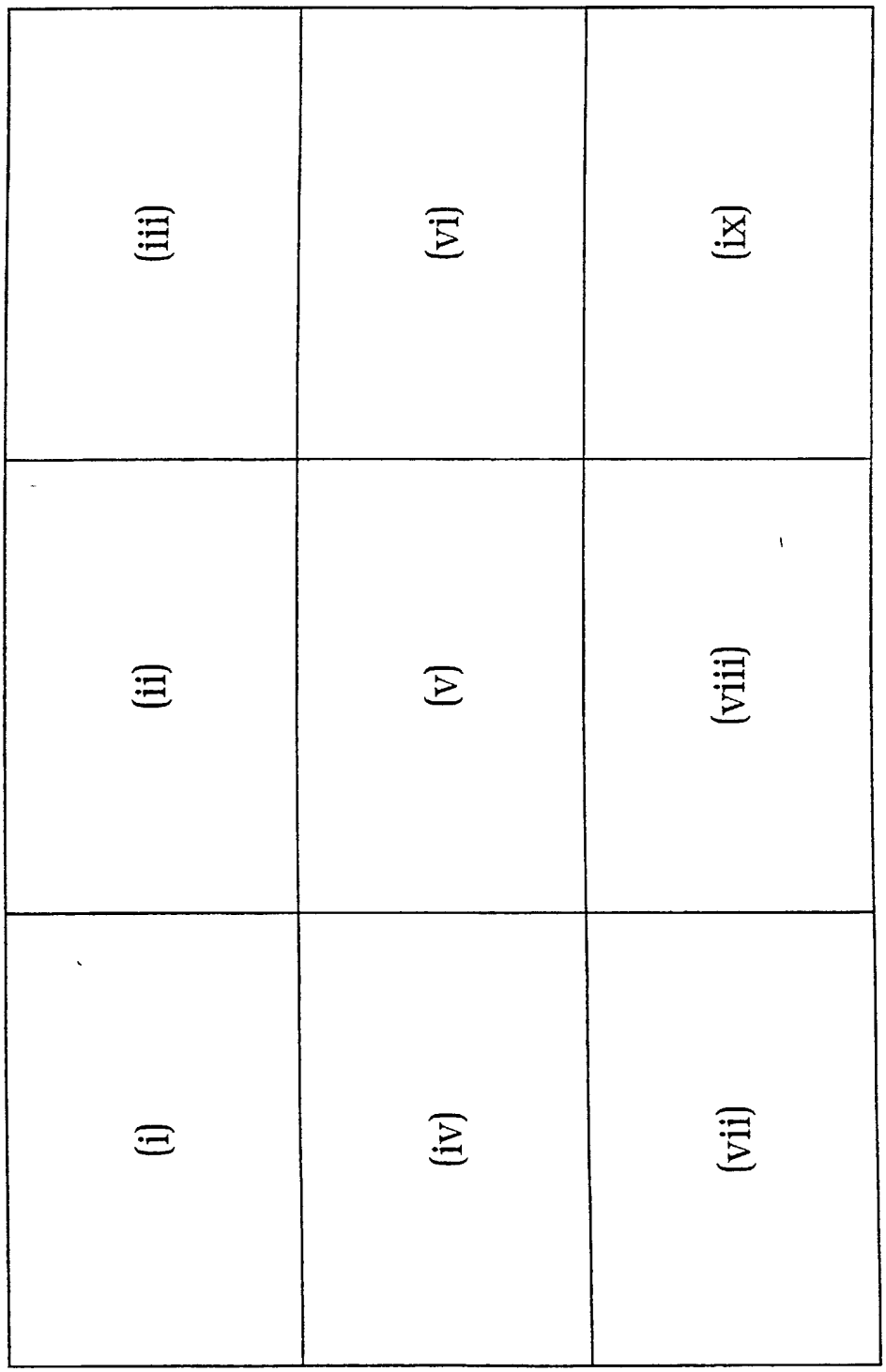
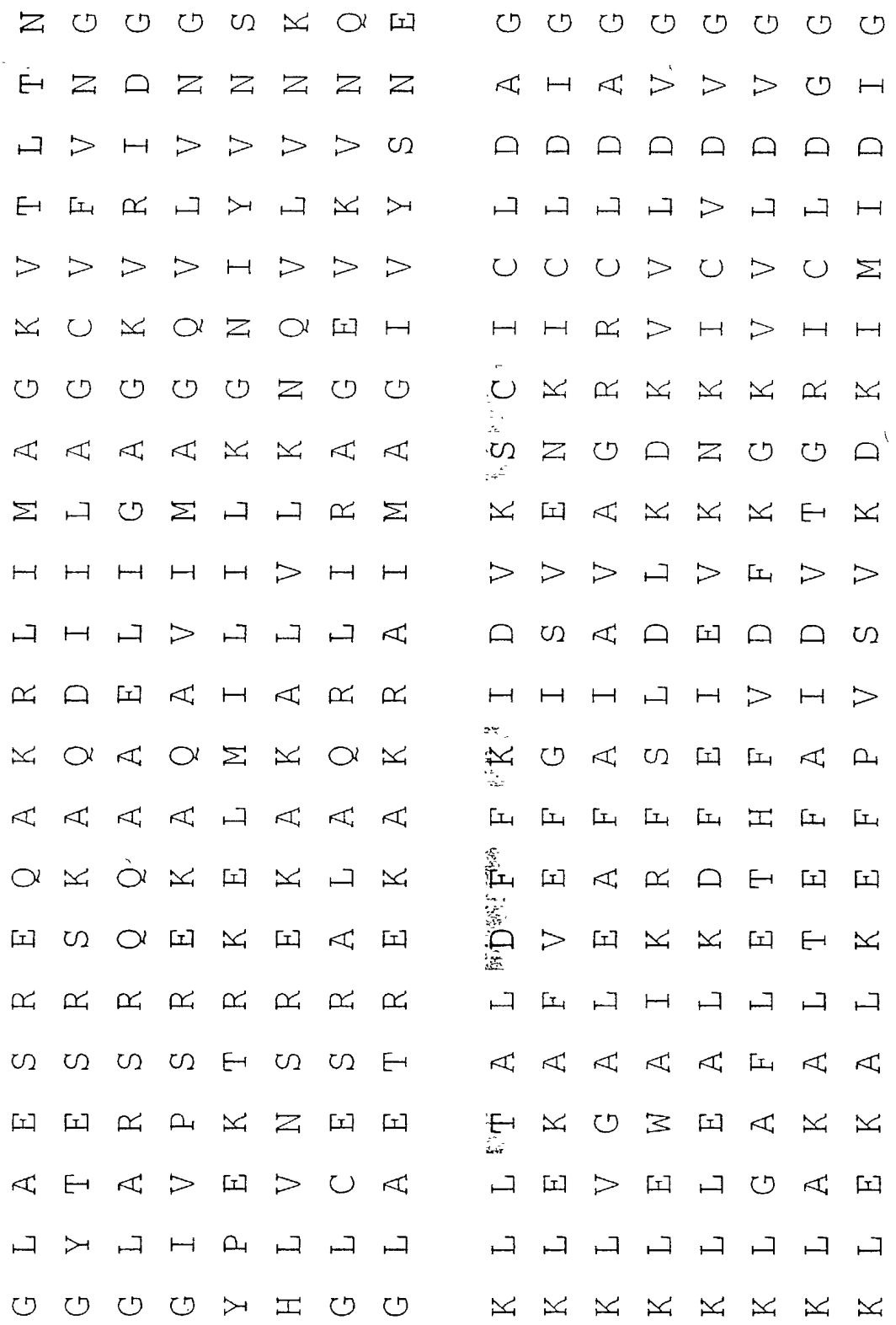


Figure 1

```
L. int  M A K - H K V R A D E L V F L Q
S. hyo  - - - - M R L D E Y V H S E
M. tub  M A - - R R A R V D A E L V R R
A. aeo  - - - - M R L D K Y L T D K
B. bur  M - K G F R N N L L N I L C K R
H. pyl  - - - - M R L D Y A L F S Q
Synec   M A K A D K Q R L D A L L V A K
B. sub  - M T S K K E R L D V L L V E R

L. int  S L I G V E R F V S R G A Y
S. hyo  E V V Q N I K - - Y V S R A G E
M. tub  T V V T D S E R A W V S R G A H
A. aeo  E V K E L P K - - Y V S R G G E
B. bur  D L V E N T C Q T F V S R G G Y
H. pyl  E L I A E K L - - F V S R A G E
Synec   E L A Q R P P - - Y V S R G G E
B. sub  T V K G N P L R - Y V S R G G L
```

Figure 1(i)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G Y L A E S R E Q A K R L I M A G K V T L T N G
G L A R S R Q Q A A Q A E L I G A G K V R I D G
G I V P K T R E K K A K A I I L K G Q V Y V N G
Y P E K T R K E L M I L L K N G N I Y V N S
H L V N S R E K K A K A L V L K N Q V L V N K Q
G L C E S R A L A Q R L I R A G E V K V V N E
G L A E T R E K K A K R L I M A G I V Y S V
K L L T A L D F F K I D V K S C I C L D A G
K L L E K A F V E F G I S V E N K I K D K G R I C C L D D I G
K L L V G A L E A F S L D L K N K G A C C L D A G
K L L E W A I K R F F E I E V K K G K V V L D V G
K L L E A L K D F E T H F V D F K V V L C V D V G
K L L G A F L E T E F A I D V T G R I C L D G G
K L A A K L T E F P V S V K D K I M I C L D G I
K L E K A L K E F P V S V K D K I D I C L D I G

Figure 1(ii)

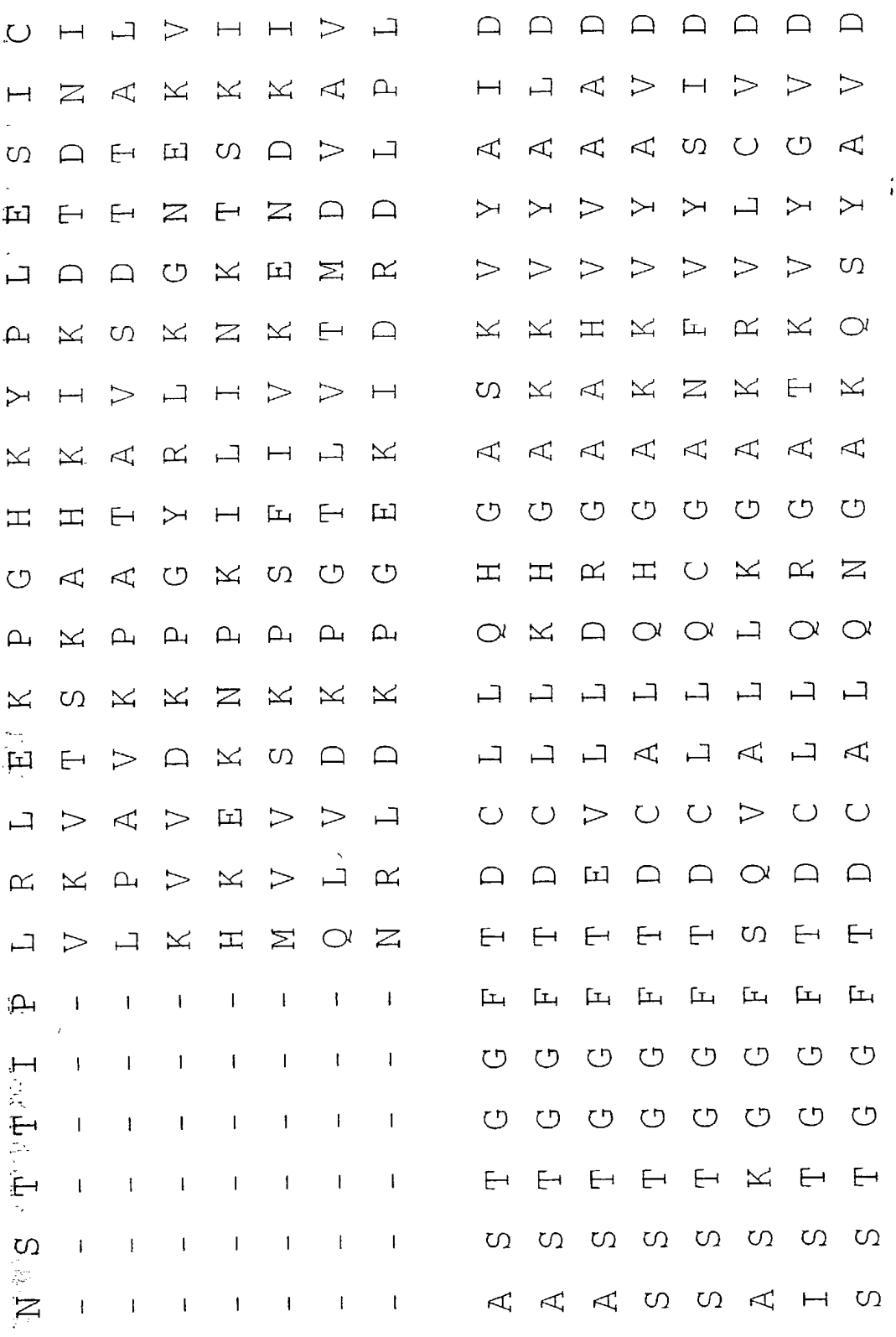


Figure 1(iii)

```
L. int  V G K G Q L H E K L Y T N E Q V     V L K A S G I I I A L I K P Q F
S. hyo  V G H N Q L V Y K L R N D N R V     E L N N L E F W V T L I K P Q F
M. tub  V G Y G Q L A W S L R N D P R V     C A S R D A D I V P L V K P Q F
A. aeo  V G R G Q M D Y K L R Q D P R V     F L K E D G L L L V L V K P Q F
B. bur  V G I N Q L S Y K L R I D P R V     K L S D N - F I I V L I K P Q F
H. pyl  V G K M Q L D E S L K Q D K R I     L S D E - - F L T L F K P Q F
Synec   V G Y G Q V A W K L R Q D D R V     L L A P P R E V V L L V K P Q F
B. sub  V G Y N Q L A W K L R Q D E R V     L L V P G S D C M A L V K P Q F
```

Figure 1(iv)

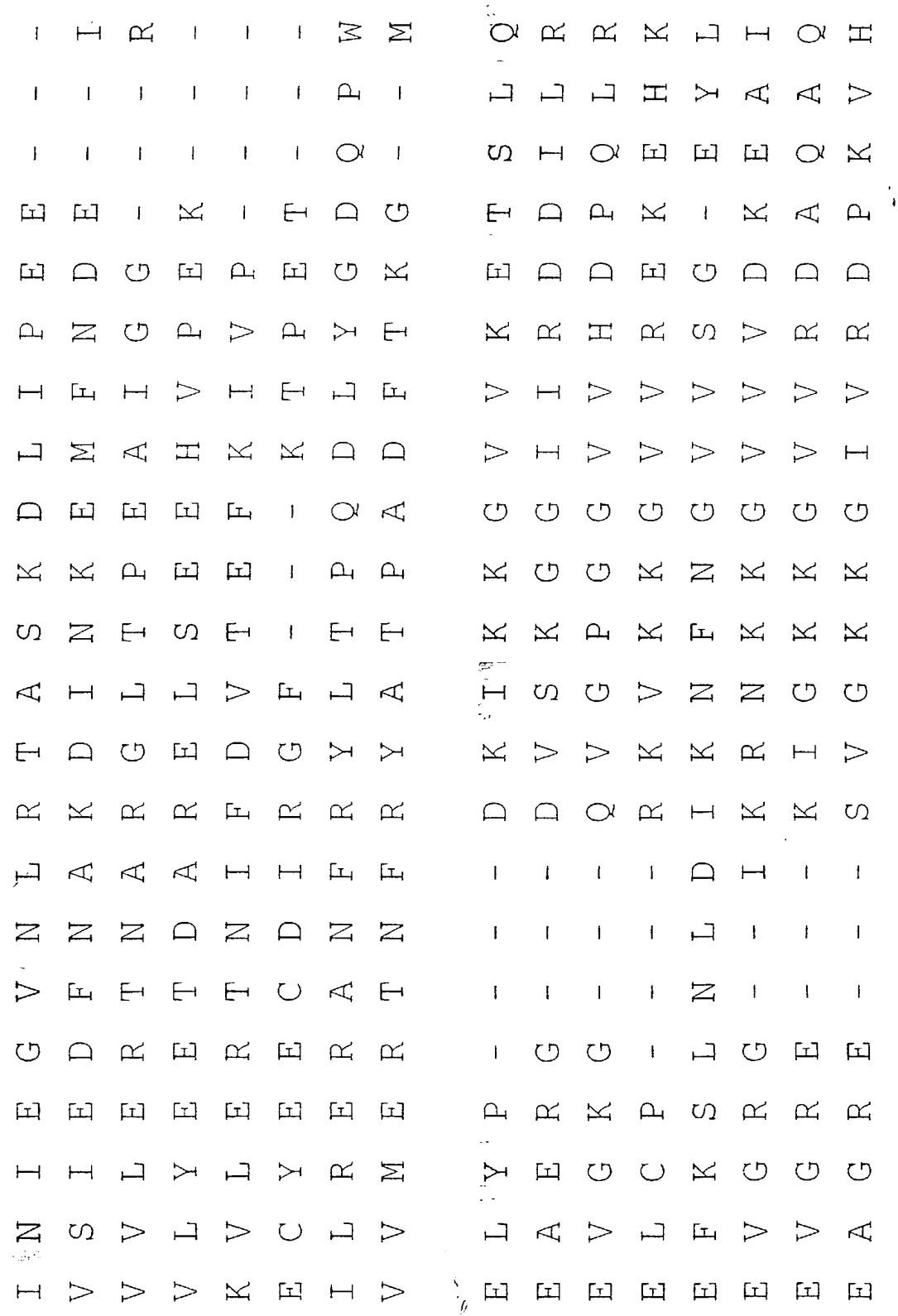
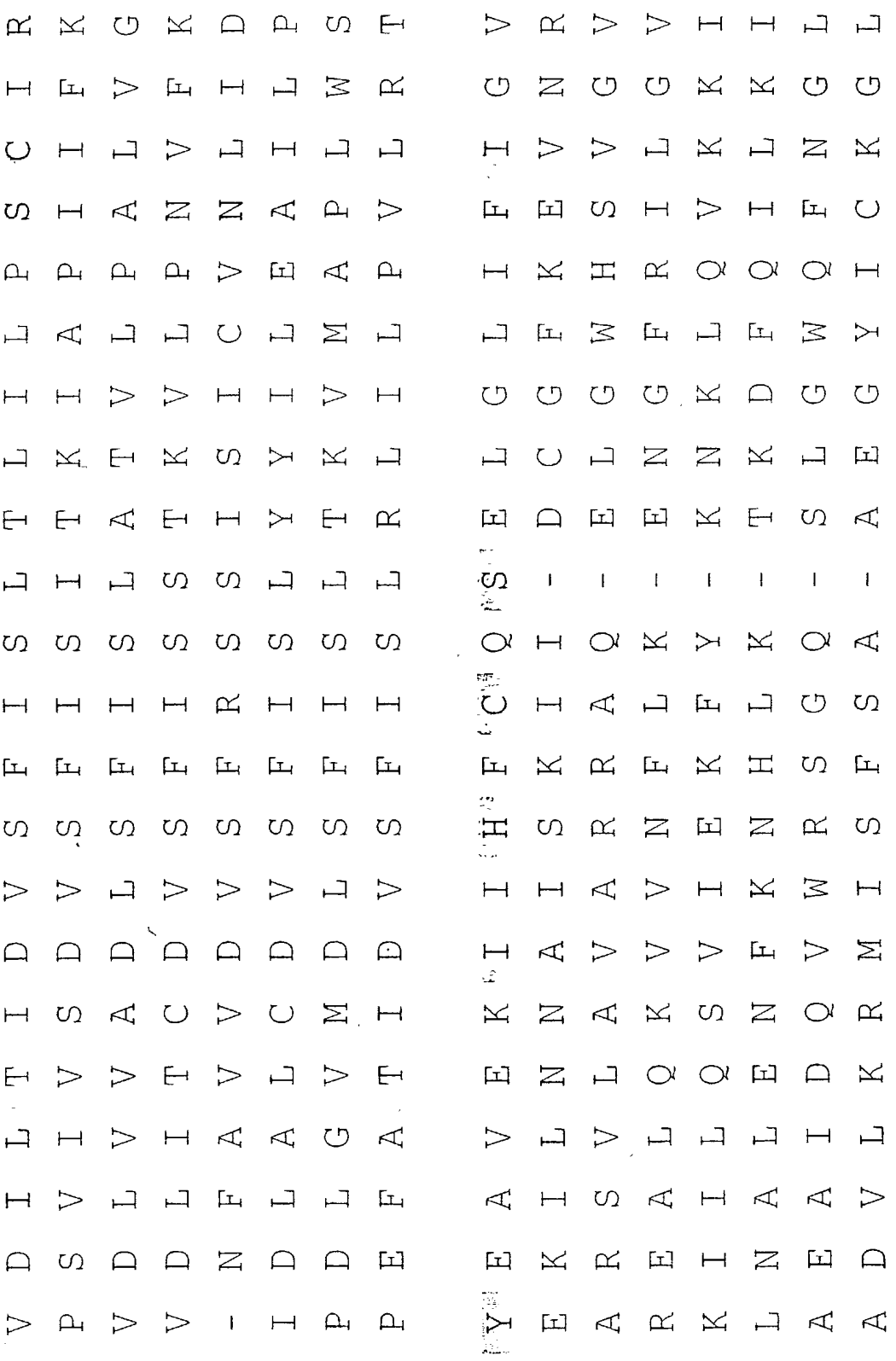


Figure 1(v)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P S V I V S D V S F I S L A T K I A P I I F K
V D L V V A D L S F I S L T V L P A L V F G
V D L I T C D V S F I S S T K V L P N V L I D
- N F A V V D V S F R S S I S I C V N L I L P
I D L A L C D V S F I S L Y Y I L E A P L W S
P D L G V M D L S F I S L T K V M A P V L R T
P E F A T I D V S F I S L R L I L P V L P V
Y E A V E K K I I H F C Q S E L G L I F I G V
E K I L N N A I S K I I - D C G F K E V N R
A R S V L A V A R R A Q - E L G W H S V G V
R E A L Q K V V N F L K - E N G F R I L G V
K I I L Q S V I E K F Y - K N K L Q I V K I
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A D V L K R M I S F S A - A E G Y I C K G L

Figure 1(vi)

```
L. int   V P S V I K G P K G N Q E Y L I
S. hyo   T I S P I K G A K G N I E Y L A
M. tub   K A S P L P G P S G N V E Y F L
A. aeo   I K S K P K G T K G N Q E F M F
B. bur   L K L K T K G K K G N V E F F I
H. pyl   Q E S L V K G K N G N V E F F L
Synec    T F S P I T G P A G N V E Y L L
B. sub   S F S P I T G G D G N I E F L L
```

Figure 1(vii)

Y	L	K	K	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	F	I	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
W	L	R	T	Q	T	D	R	A	L	S	-	-	A	K	G	L	E	D	A	V	H
L	A	G	R	K	G	E	E	V	N	L	S	-	-	E	A	I	E	K	A	L	E
L	V	V	K	S	S	V	L	R	I	A	S	S	M	Q	L	L	S	N	I	E	F
H	F	K	R	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
W	L	S	Q	S	A	V	N	A	S	A	P	T	L	A	Q	L	G	E	I	T	Q
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Figure 1(viii)

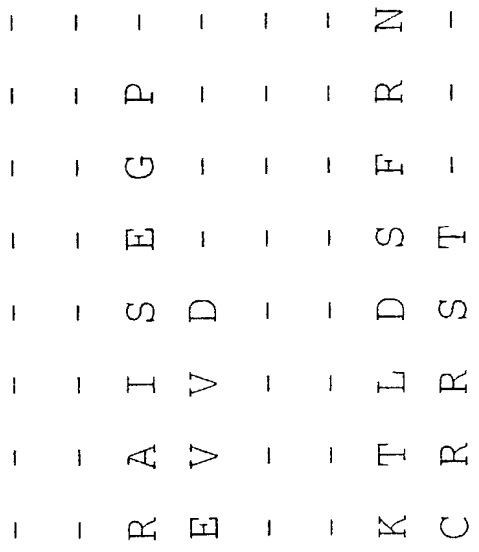

Figure 1(ix)

Page 1 Attorney's Docket No. _____

DECLARATION AND POWER OF ATTORNEY - USA PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled LAWSONIA DERIVED GENE AND RELATED HEMOLYSIN POLYPEPTIDES, PEPTIDES AND PROTEINS AND THEIR USES the specification of which:

(a) ☐ is attached hereto; or

(b) ☐ was filed on _____ as ☐ Application No. 0 /_____ or ☐ Express Mail No., as Application No. not yet known _____ and was amended on _____ (if applicable); or

(c) ☒ was described and claimed in PCT International Application No. PCT/AU00/00439 filed on 11 May, 2000 and as amended under PCT Article 19 on _____ (if any) and/or under PCT Article 34 on _____ (if any).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above;

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56;

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent, design or inventor's certificate or any PCT international application(s) listed below and have also identified below any foreign application(s) for patent, design or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed for the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

COUNTRY (OR INDICATE IF PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 37 U.S.C. § 119
PCT	PCT/AU00/00439	11 May 2000	☒ YES NO ☐
			☐ YES NO ☐
			☐ YES NO ☐
			☐ YES NO ☐
			☐ YES NO ☐

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below, and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

KNOBBE, MARTENS, OLSON & BEAR, LLP
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(714) 760-0404 FAX (714) 760-9500

Prior U.S.A. Application(s)

Application No.: 60/134022 Filing Date: 13 May, 1999 Status: _____

POWER OF ATTORNEY: I hereby appoint the registrants of Knobbe, Martens, Olson & Bear, LLP, 620 Newport Center Drive, Sixteenth Floor, Newport Beach, California 92660, Telephone (714) 760-0404, Customer No. 20,995, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith (if this application is assigned, I acknowledge that the appointed individuals do not represent me, and that instead they represent the assignee).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

100

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Inventor's signature Panaccio Day ___ Month _____ Year _____

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200

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Full name of fifth inventor: _____

Inventor's signature _____ Day ___ Month _____ Year _____

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Citizenship: _____

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Full name of sixth inventor: _____

Inventor's signature _____ Day ___ Month _____ Year _____

Residence (city and country): _____

Citizenship: _____

Post Office Address: _____

Full name of seventh inventor: _____

Inventor's signature _____ Day ___ Month _____ Year _____

Residence (city and country): _____

Citizenship: _____

Post Office Address: _____

Full name of eighth inventor: _____

Inventor's signature _____ Day ___ Month _____ Year _____

Residence (city and country): _____

Citizenship: _____

Post Office Address: _____

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